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ENDOCRINOLOGY

VOLUME 43 JULY, 1948 NUMBER 1

PERIODICITY IN BODY TEMPERATURE AND HEART RATE

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CHICAGO, ILLINOIS

IT HAS been known for over a century that the human body temperature is not constant, but exhibits regularly recurring fluctuations. A diurnal, or 24-hour, cycle is related to the influence of the daily routine of work, meals, rest, and sleep. A longer cycle in menstruating women has been established more recently, but there are no consistent data on a seasonal or annual temperature rhythm in either sex. The heart rate, which is much more labile than body temperature, has also been found to show a diurnal variation, but figures pertaining to longer-term periodicity are few and contradictory. Furthermore, the relation between body temperature and heart rate, although very definite in cold-blooded animals, as well as in hyperthermia in man, has not been clearly demonstrated in the latter under normal conditions of every-day existence. It is our purpose to present observational data on human body temperatures and heart rates which bear on their diurnal, menstrual and seasonal periodicity; the individual form and constancy of the several cycles; the endogenous and exogenous factors that influence them; and, finally, the close interrelation between the two variables studied.

The tools employed were the ordinary clinical thermometer and the second hand of a watch or clock. Most of the data were obtained by self-observation. The subjects established a routine of existence that they tried to maintain for months, or even years (with occasional breaks for vacations), and those who were temperamentally unfit for regular hours, or wearied of gathering figures, dropped out. For this reason, and also because it takes some weeks to adjust oneself to the life of a "human guinea-pig" the study was intensive, rather than extensive. In all, about two dozen subjects were studied, but this report

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is based on data on ten subjects, six male and four female. Of the male subjects, NK, JN, and HF were middle-aged, and AR, JM, and ML, younger adults; of the females, RN and EH were young adults, and HK and EK, teen-age sisters.

For the diurnal cycle, mouth temperature and radial pulse were determined at two-hourly intervals during the waking hours, and once or twice during sleep, the subject being aroused by an alarm clock set for a definite hour. The thermometer was kept under the tongue for a full five minutes, then read to the nearest 0.05°F., and the pulse counted during this period until two successive half-minute counts showed no difference. The observations were made in the sitting or lying position, after at least 15 minutes of rest. Precautions were taken not to expose oneself to outside air during the winter months for half an hour prior to making temperature readings. Likewise, hot and cold liquids were avoided for an equal length of time. By a simple code-marking system, the subjects indicated on their temperature and pulse charts the incidence of meals, taking of tea or coffee; activities, such as work or recreation (card playing, movies, etc); the occurrence and duration of day-time naps, and the time of going to bed and getting up. Meals were timed to follow rather than precede observations. As a longer time unit, the week was found more suitable than the month, since most of the day-to-day variations in routine follow the days of the week. For the menstrual cycle, the day of the onset of menstruation (M) was adopted as the zero point, from which days were counted backward as well as forward. Figures obtained from October to March were applied to winter, while May to August was considered the summer season. For statistical treatment the "t" method of Fisher (1941) was employed, and differences were designated as significant when the probability of their being due to chance was less than one in a hundred ($P<0.01$). Data for each individual were usually analyzed separately, and attempts were made to bring out not only similarities, but also differences among the several subjects.

I. *The diurnal cycle of body temperature.* The existence of this cycle hardly needed confirmation, but the many thousands of temperature readings we have accumulated permitted us to determine the time of incidence and the constancy of the maxima and minima, as well as the degree of dispersion or concentration of the values pertaining to particular phases of the various cycles. In the several parts of fig. 1 and upper part of fig. 3 are histograms, arranged horizontally, for the frequency with which certain temperature readings occur at different hours in individual subjects. The ranges of readings for almost any hour often exceed the range of the mean diurnal body temperature curve. Yet, distinct modes can usually be made out in frequency distribution curves, formed by the right-side ends of the histograms. The 6 A.M. temperatures of parts A and B of fig. 1, pertaining to one person, show at once the least dispersion and greatest resemblance to

the conventional probability curves. Although the mean maxima of both curves are at 2 P.M. and minima at 2 A.M., many 2 P.M. temperatures are lower than some 2 A.M. values. On the contrary, parts C and D, also of one individual, show a much greater concentration of

FIG. 1. Frequency distribution histograms of mouth temperatures of 6 subjects, all male, as determined for the different hours of the day and night. The vertical temperature scale is the same for all eight parts, the horizontal (number of readings for each temperature value) is to be referred to the length of the bar, next to each letter, which length represents 20. The succession of hours is not to be considered a scale, as these hours are only reference points to the sets of bars above them. *A*, 3192 temperature readings, 10 per day, of NK, during the summers (mid-May to mid-October) of 1941, 2, and 3; *B*, 3352 readings for the same subject, during the winter months (October to March) of 1941, 2, 3, and 4; *C*, 3254 readings, 12 per day, of AR during the summers (June to August) of 1941, 2, and 3; *D*, 3236 readings for the same subject in the winters (December to February) of 1940, 1, 2, 3, and 4; *E*, 656 readings, 9 per day, of HF from March to May of 1942; *F*, 1019 readings, 9 per day, of JH from February to August of 1942; *G*, 357 readings, 9 per day, of JM from February to April of 1942; and *H*, 2962 readings, 10 per day, of ML from June 1942 to June 1944. Total number of temperature readings, over 18,000; number of diurnal cycles, over 1,800.

temperature values for every hour of the day and night. In C all the temperatures taken from 11 A.M. to 7 P.M. are higher than all the temperatures from 11 P.M. to 6 A.M. The maxima of both C and D are at 3 P.M. ,while the minima occur at 3 A.M. giving a 12 hour separation of the two, as in parts A and B. Frequency distribution curves of the incidence of the maxima and minima, not reproduced here, also resemble probability curves, with a greater dispersion of the hours of individual daily maxima than of the daily minima. This results in a

smoother peak and a sharper trough of the diurnal curves, seen even more clearly in the mean diurnal temperature curves for the same subjects in the right-side portions of fig. 2. Part G of fig. 1 resembles parts A to D, even though it is confined to the waking hours and is based on a rather small number of days. The mean maximum is

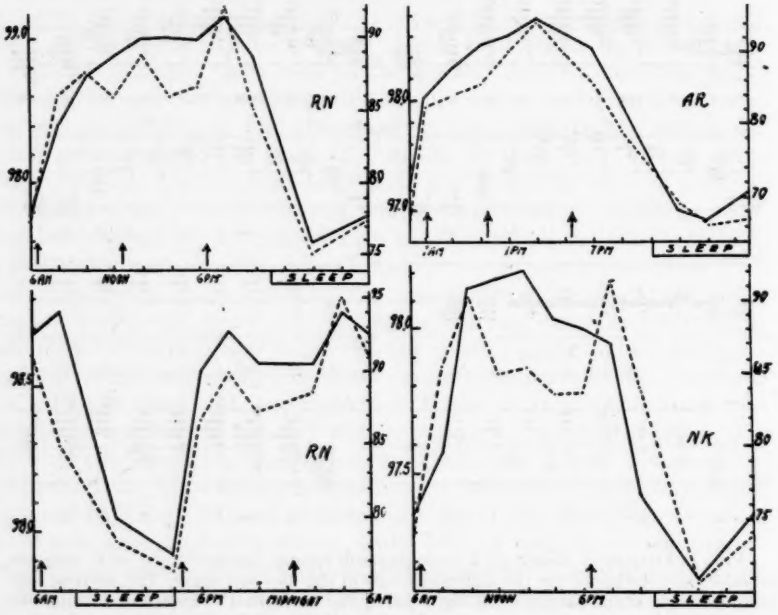

FIG. 2. Mean body temperatures and heart rates for the successive hours of the diurnal cycle. Solid lines, body temperatures, scales on the left of the curves; broken lines, heart rates, scales to the right. Arrows indicate usual meal times. Upper left, data for 168 days, January to June, 1944, 1° corresponding to 10 heart beats; lower left, data for 126 days, June to October, 1944, 1° corresponding to 20 heart beats. The frequency distribution of the temperatures upon which the means in both left-hand figures were based are shown in fig. 3. Upper right, data for 175 days, May to October, 1943, and lower right, for 224 days, May to October, 1943.

98.81°, falling at 2 P.M., and the rise is somewhat steeper than the fall.

A different type of curve is shown in part H of fig. 1. This person's temperature modes are all the same from noon to 4 P.M., although the 2 P.M. mean is the highest, but the maximum daily temperature is reached only at 8 P.M. The minimum is 4 A.M. (not shown in the figure), the maximum-minimum interval being about 8 hours. A similar relationship is found in the upper part of fig. 3, where there is little change in temperature from 2 to 6 P.M. (also pictured in the upper left part of fig. 2), but the maximum is at 8 P.M. and the mini-

mum at 2 A.M., or perhaps a little later. In any case, the maximum-minimum interval is about six hours, resembling part H of fig. 1 in this respect also. We thus have four individuals whose diurnal temperature minima occur at about the same time, during sleep, while the maxima are reached either in the afternoon or in the evening, but are constant for the particular person.

A third type of curve is seen in parts E and F of fig. 1. Instead of

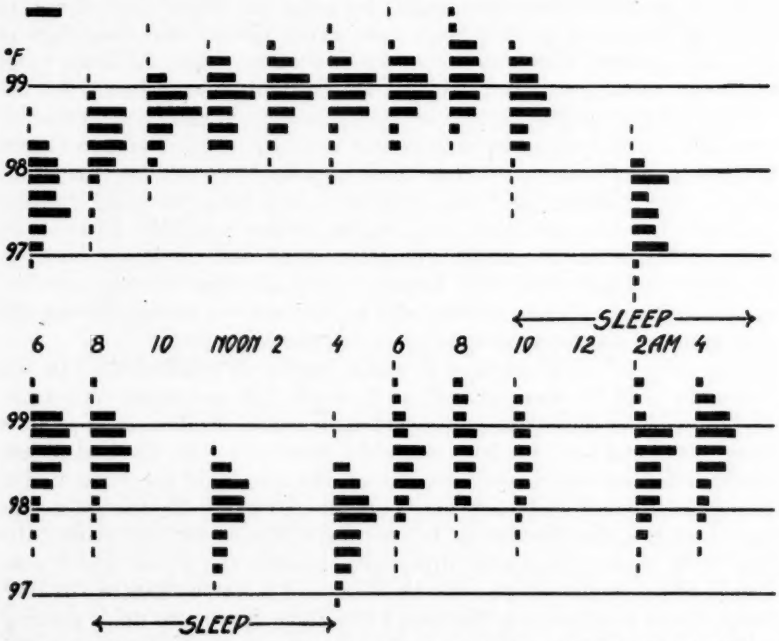

FIG. 3. Frequency distribution histograms of mouth temperatures of RN, female, arranged in the same way as in fig. 1. The mean values of these temperatures are plotted in the solid lines of the left-hand parts of fig. 2.

a peak, there is a plateau, which is quite distinct in E, and almost extreme in F. In E the morning rise is small, and the modes are the same, 98.0°, from 8 A.M. at 8 P.M. The mean temperatures do not vary more than 0.02° from noon to 4 P.M. In F the rise is sharper from 6 A.M. to 10 A.M., but thereafter the mode is the same, 98.6°, till 10 P.M. From noon to 8 P.M., the mean temperatures do not vary by more than 0.07°. This is the only subject whose temperature was the hypothetical 98.6° in about 40% of all the readings made between 10 A.M. and 10 P.M.

II. *The diurnal cycle of heart rate.* Though one of the most labile of physiological values, the heart rate figures arranged in frequency

distribution histograms show the same picture as do body temperatures in figs. 1 and 3, and are therefore omitted. However, the mean diurnal curves of heart rates are usually less smooth than the corresponding temperature curves (fig. 2), and in most subjects the heart rate tends to rise sharply after meals. In the upper left part of fig. 2 there are three such post-prandial rises, while in the lower right part of the same figure, with only two meals per day, there are two sharp heart rate peaks, at 10 A.M. and 8 P.M. In the upper right part, however, a complete parallelism exists between the mean diurnal curves for body temperature and heart rate, every upturn and downturn in one being closely followed in the other. By and large, the heart rates of these, as well as other subjects studied, all show distinct diurnal curves, and by adjusting the scales, these curves can be seen to correspond to the body temperature curves, with a swing of one degree F. involving a change of 10 to 20 heart beats per minute, the exact equivalents varying with the individual and with the daily routine followed. These equivalents are roughly 20 beats in NK, 15 beats in AR, 10 beats in RN under regular routine and 20 beats under a schedule of night work and daytime sleep. Further correlations between the two variables studied will be brought out in considering the endogenous and exogenous influences on the organism.

III. *The effects of changes in daily routine.* A marked shift in the hours devoted to sleep, as well as to work, led to a rapid change in the diurnal temperature and heart rate curve of RN. In a week's time, her mean lows for both variables were at 4 P.M., the end of her sleep period, as contrasted with 2 A.M., the middle of the night under the usual routine (left top and bottom of fig. 2). However, if one examines the distribution of temperature figures for the same subject in fig. 3, one finds little difference between the 2 A.M. and 6 A.M. values of the night sleep, and the noon and 4 P.M. values of the day sleep. More significant is the rapid rise in temperature upon getting up at 4 P.M., compared to a much slower one on a daytime work routine (figs. 2 and 3). The 8 P.M. high, although exceeded slightly by the 4 A.M. and 8 A.M. peaks, still corresponds to the usual 8 P.M. maximum, thus showing a failure to obliterate completely the usual temperature cycle, in spite of a relatively prompt change-over of the diurnal temperature curve to conform to the new routine. Another important difference between the mean temperature curves of RN is the range of 1.57° under usual conditions and one of 0.83° with daytime sleep. Yet, a glance at fig. 3 reveals that the over-all distribution of temperature figures is about the same under the two regimes, about 3°, with only some 2 A.M. temperatures in night sleep falling much below 97°. The difference in the mean curves may be connected with sleep under the shifted routine occurring during the warmest part of the diurnal cycle and wakefulness during the coolest, but the instability of the temperature cycle, as reflected in the lack of distinct temperature modes for 6, 8 and 10 P.M., following daytime sleep, is

also a factor. By contrast, the adaptation of the heart rate cycle to the new conditions is very good, the post-prandial peaks appearing in the proper places (except after the 6:30 A.M. "supper" which was closer than usual to bed-time), the mean diurnal range, 28.8 beats, not differing from the normal range of 27.1 beats.

Smaller shifts in routine, of 3 or 4 hours, lasting from 6 to 10 weeks at a time, were made by AR and NK. Fig. 4 shows a mean weekly "getting up" temperature of 97.2° for AR after arising at 3 A.M. for a number of weeks. Changing the hour of getting up to 6 A.M. raises the basal temperature, with a gradual descent to the previous figures.

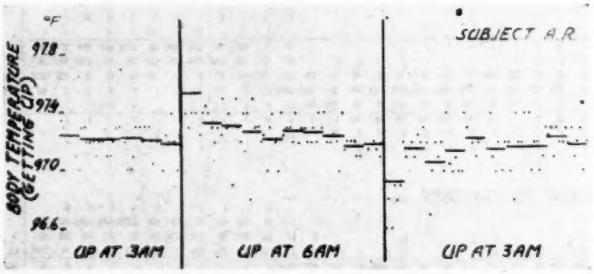

FIG. 4. Basal, or "getting-up," temperatures for 182 consecutive days of AR. Dots represent individual temperature readings, bars, the weekly means.

The same sort of effect, but in the opposite direction, is produced by once again shifting the getting up hour to 3 A.M.

The mean diurnal body temperature range was only slightly increased (from 1.86° to 1.93°) during these shifts for AR, but in the case of NK, during two six-week periods of going to bed between 2 and 3 A.M. and three similar periods of going to bed between 6 and 7 P.M., the mean diurnal temperature range was 1.62°, compared to a mean range of 1.45°, when going to bed at the usual time (about 11 P.M.). The daily maxima and minima were more scattered during shifts than under the normal schedule, with the former tending to be bimodal. On the whole, a better adjustment of the body temperature curve to the new schedule occurred when the onset of sleep was 4 hours later than usual, after 2 A.M., than when it was set 4 hours earlier, after 6 P.M.

IV. *The menstrual cycle.* Data were first gathered on the two sisters (HK and EK) each of whom started taking evening and morning mouth temperatures shortly after she began to menstruate. Fig. 5 shows the frequency distribution of temperatures for successive days of the menstrual cycle, counting back from the first day of menstruation (M) for HK, and a similar distribution, not illustrated, had been obtained for EK. The outstanding feature is the almost complete parallelism between the evening and morning temperatures, the

former a little over one-half degree higher. There is a slow decrease during the first half of the cycle, with a mean low of 98.18° on the evening of day −16, and a low of 97.59° on the morning of day −15. Then there is a sharp rise with almost a plateau till two days before the onset of menstruation, when there occurs a sharp dip which is carried over into M day and beyond. The temperature ranges of the mean menstrual curves amounted to 0.74° (evening) and 0.76° (morning) for this subject, but individual swings were usually greater, as ovulation which coincides, or shortly follows, the low point of the

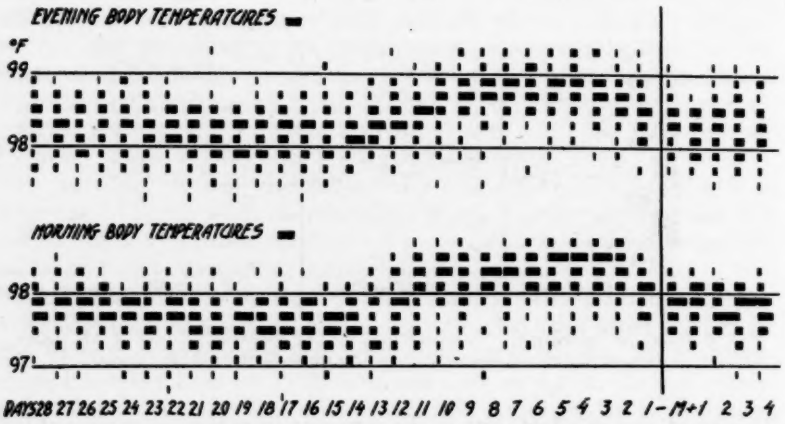

FIG. 5. Frequency distribution histograms of daily mouth temperatures of HK before going to bed at night and immediately upon getting up in the morning, arranged with respect to the first day of menstruation (M). Horizontal bars to the right of each title represent 10 temperature readings. Data start with the second cycle after the onset of menstruation and are based on 34 periods, between Nov. 11, 1941 and Nov. 26 1944. As some of the early menstrual cycles were apparently anovulatory, their exclusion from this series would make the temperature mid-period rises much steeper.

cycle did not always occur on the same day. Thus, an analysis of the 34 cycles whose data were used for fig. 3 shows that the low evening temperature occurred between day −20 and day −13, with a mean of −15.3. The individual evening temperature swings of the several cycles varied from 0.70° to 2.15°, the mean range amounting to 1.19° (97.34°–98.53°). Although the mean morning temperature range was almost exactly the same, 1.20° (97.93°–99.13°), the latter varied only from 0.60° to 1.65° for individual cycles, and the statistical significance of the mean morning range was higher. The inconstancy of the day of ovulation is also shown by ranges of the mean temperature curves of the other three subjects, as compared with their mean swings: the former are 0.65°, 1.05°, and 0.83°, while the latter are 1.03°, 1.22°, and 1.12° for EK, EH, and RN, respectively. In fig. 6, the composite

basal, or getting-up, temperatures and the corresponding heart rates are shown for these three subjects, as grouped about the low temperature day (O) of the cycle and the first day of menstruation (M). It can be seen that the menstrual temperature curve is monocyclic and fairly smooth, while the heart rate curve is definitely dicyclic and jagged. With respect to O day, the heart rate rises steeply on +1 day and drops almost as much on +2 day, whereas the body temperature continues on its upward course, though in progressively

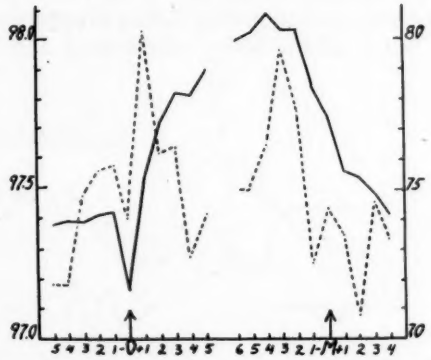

FIG. 6. Composite mean curves of basal mouth temperatures and heart rates of three subjects. The day of low temperature, followed by a steep rise, in each cycle was arbitrarily designated as O day, and the data for − and + days were arranged to straddle this day of supposed ovulation. M day is the first day of menstruation and was therefore fixed for each cycle. Out of a total of 37 cycles, whose values furnished the means for these curves, there were 13 of EK (Feb. 6, 1947 to Jan. 13, 1948), with O day varying from −8 to −13, and a mean of −10.8; 13 of EH (Nov. 12, 1945 to Nov. 13, 1946), with O day varying from −14 to −18, and a mean of −16.3; and 11 of RN (Jan. 1 to Nov. 27, 1944), with O day varying from −11 to −16, and a mean of −13.8. The breaks in the curves are necessitated by the inequality in the intervals separating O and M days in the different subjects, and in the several cycles of each subject.

smaller increments. This combination of heart rate and temperature changes makes it possible to fix the location of O day in the cycle with greater accuracy than by temperature figures alone. A second peak of heart rate, higher or lower than the first, is reached before menstruation, and is followed by a sharp drop, coinciding with, or slightly preceding, the pre-menstrual body temperature fall.

For RN, who took her temperature and heart rate 10 times daily (fig. 3), the mean daily temperatures and heart rates, which we call the temperature and heart rate *levels*, fall into the same curve with respect to O and M days, as do her basal values. In other words, the menstrual curves are the same, whether based on one, two, or ten daily readings. There seems to be an exogenous, probably nervous, influence on the onset of menstruation, as has been pointed out by many writers. Of the two sisters studied, the older, HK, had a mean

menstrual cycle of 32.6 days for the twenty periods, from November, 1941 to September, 1943, before EK began to menstruate. From then until the end of 1945, HK had 26 cycles, whose mean duration was exactly the same, 32.6 days, while EK had 27 cycles, with a mean of 28.7 days. The difference in duration, 3.9 days, was highly significant, and days of onset were often far apart. From January to November of 1946, however, each girl had 10 cycles, with means of 29.1 and 28.7 days respectively, and, in addition, the days of onset of menstruation were, on the average, only 3 days apart.

Menstrual periodicity is abolished during pregnancy and lactation, but each of the latter affects body temperature and heart rate. Al-

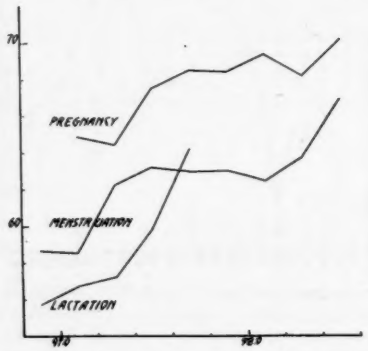

FIG. 7. The relation between mean basal body temperatures and heart rates of EH during pregnancy (Dec. 10, 1944 to June 26, 1945), subsequent period of non-menstruation (July 12 to Nov. 11, 1945), and one year of 13 menstrual cycles (Nov. 12, 1945 to Nov. 12, 1946). Both temperatures and heart rates are higher in pregnancy and lower in lactation than they are during regular menstruation cycles.

though our results are limited to a single pregnancy and subsequent lactation (or, at least, non-menstruation period), they are clear-cut and quite marked. As shown in fig. 7, pregnancy figures for both temperature and heart rate were highest, those for lactation, lowest. That seasonal differences were not responsible was found by dividing the menstruation data into two portions, one to conform seasonally to pregnancy and the other to lactation, and plotting them separately. While the graphs crisscrossed, they were essentially superimposable and just as distinctly below the pregnancy curve and above the lactation one, as is the combined menstruation curve in fig. 7. Further treatment revealed that for a range of 97.4° to 98.4°, comprising 78% of all the pregnancy temperature figures, the mean heart rate was 68.4; for a range of 97.2° to 98.4°, making up 87% of menstrual cycle temperatures, the mean heart rate was 62.9; and for a range of 96.8° to 97.4°, or 76% of the lactation temperatures, the mean heart rate was 56.5. The higher value of the mean heart rate during lactation

when the basal temperatures were 97.6° to 97.8°, as compared to the mean heart rate during the menstruation cycles for the corresponding temperature range, is easily explained by the dicyclic heart rate curve and the monocyclic temperature one during each menstrual cycle, which tends to make the heart rate curve almost a horizontal line between 97.4° and 98.2°.

V. *Seasonal Periodicity.* Among the exogenous influences on body temperature, and thus indirectly on heart rate, there is the seasonal

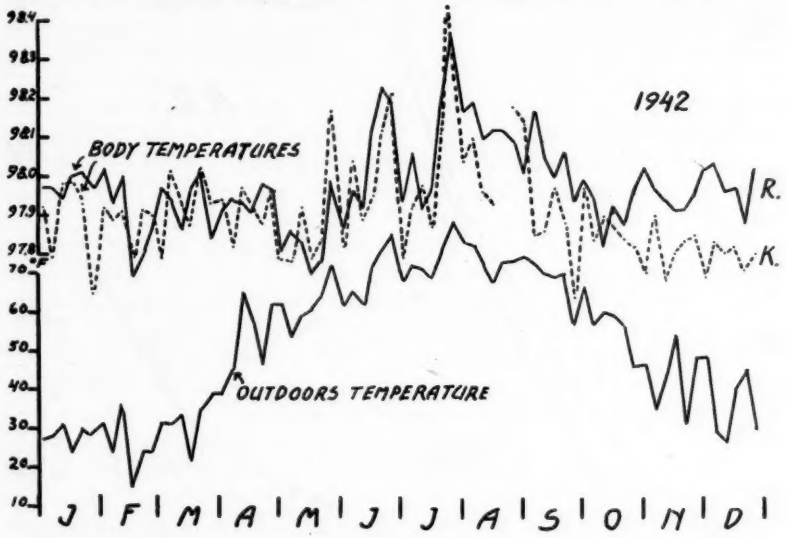

FIG. 8. The variations in successive 5-day body temperature levels of two subjects during 1942 and fluctuations of outdoor temperature levels for corresponding periods.

variation in external temperature. Lest this appear contradictory to the statement that there was essentially no difference in the temperature and heart rate figures for the different seasons in the menstruation curve of fig. 7, it should be pointed out that the latter applied only to "getting-up," or *basal*, values. Indeed, a reference to parts A and C of fig. 1, representing summer body temperatures and parts B and D of the same figure for winter data, will reveal that the 6 A.M. temperatures are the same for both seasons and, as already indicated, have the most prominent modes, 97.2° to 97.4°. for both NK and AR (the same can be said about the basal heart rates, not shown in figure). Otherwise, the occurrence of temperatures over 99.0° is more frequent for both subjects, and the modes are less prominent, in the summer. In the winter, when the inside temperature is kept at a fairly uniform level of about 70°, one lives in an artificial climate, and external temperature variations have no chance to impose their ef-

fects on body temperature under the conditions prescribed for making observations.

To bring out the effects of external temperature we determined the mean of the 10 or 12 observations made at the different hours, which, as mentioned, is designated as the temperature level for the whole 24 hours. Fig. 8 shows the fluctuations of such body temperature levels,

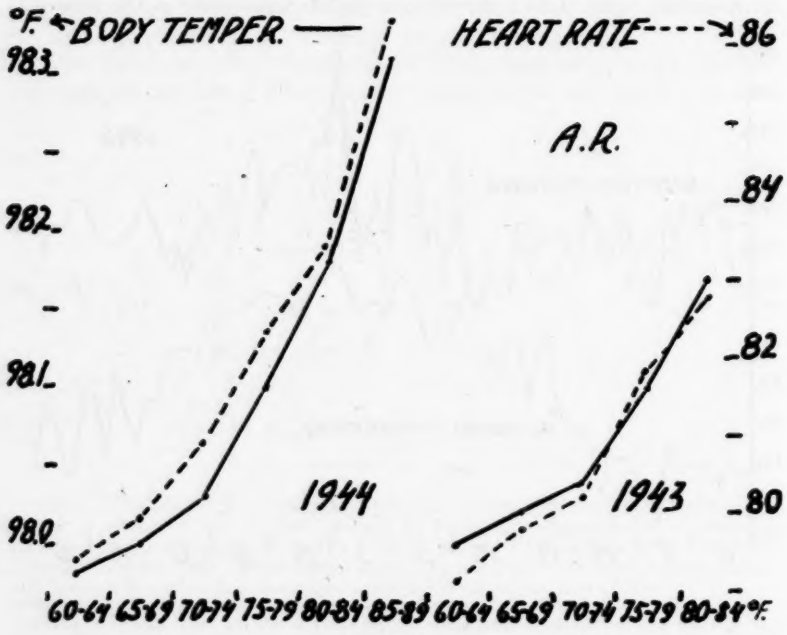

FIG. 9. Mean body temperature and heart rate levels of AR for days when the outdoor temperatures fell into the categories shown in the abscissae, for 1943 and 1944. The absence of a 85–89° interval in the 1943 graphs was due to a cooler summer in that year.

means of successive 5 days of each month throughout 1942, for AR and NK. It can be seen that the two body temperature levels rise and fall, independently of each other and of outdoor temperatures during the cold months of the year. Only between the middle of May and the end of September, the period when no artificial heat is supplied, is there a parallellism among the three curves, the warmest five-day stretch of outdoor temperatures corresponding to the highest body temperatures for both subjects. Furthermore, from March to May, when the external temperatures were going up, and after September, when they began to fall, the body temperatures were no different than during January, February, and March. We have similar curves for

other years, as well as for heart rate levels, obtained in the same manner as temperature levels. The relationship between the external temperature and the levels of heart rate and body temperature is shown in fig. 9. The temperature and pulse values for outdoor temperatures from 20° to 60° were omitted as they lie on an essentially horizontal line, both variables beginning to rise, first gradually, and then steeply, only after the outdoor temperatures exceed 60° ("no-heating" season).

Similar curves can be drawn from the data on body temperature and heart rate levels available for NK and RN. These data also afford an opportunity to determine by how many beats the pulse is increased as the body temperature rises with the external temperature. From fig. 9 it can be seen that, for AR, in 1943 a rise in temperature level of 0.17° entails an increase in heart rate of 3.7 beats per minute, corresponding to 21.8 beats per degree F. In 1944, for the same subject, a temperature swing of 0.34° is accompanied by a rise in pulse of 6.9 beats, or 20.3 beats per degree F. Correlation coefficients between the changes in body temperature and those in heart rates are very high, 0.998 and 0.972 for the two years. The week-to-week fluctuations of these values for AR show correlation coefficients of 0.723 and 0.665, both highly significant, for the same two years. The body temperature and heart rate changes for NK, arranged as in fig. 9, give a rise of 8.1 heart beats for 0.48° (correlation coefficient of 0.957), or 16.9 beats per degree F. His week-to-week fluctuations of body temperature and heart rate levels show a correlation coefficient of 0.772. In both subjects, the number of heart beats per degree F. change in body temperature is much higher for lower external, and therefore lower body, temperatures, when the figure may be as high as 35 beats per degree F.

In the mean diurnal body temperature and heart rate curves, shown in fig. 2, on the contrary, AR shows a change of about 15 beats, and NK of 20 beats, per degree F, whereas for RN the number is about 10 beats per degree F. under the usual routine, and 20, when she slept in the daytime. Incidentally, her week-to-week fluctuations of body temperatures and heart rate levels had a correlation coefficient of 0.570 ($P < 0.01$), not as good as those for the male subjects, but still reflecting the effect of the external temperature variations. Again, the corresponding *basal* temperatures and heart rates had a correlation coefficient of 0.370, which was not statistically significant.

VI. *The effects of thyroid.* For a further test of the interrelation between body temperature and heart rate, NK and AR took two grains of desiccated thyroid daily for fourteen days, followed by another 14 days of no thyroid. Basal metabolic rates and body weights were measured at the end of each week. The results were similar for the two subjects, and in fig. 10 can be seen the mean daily levels of body temperatures and for heart of NK, based on 10 successive 28-day periods. The mean metabolic rate went up during the period of thyroid intake, more in the first than in the second week,

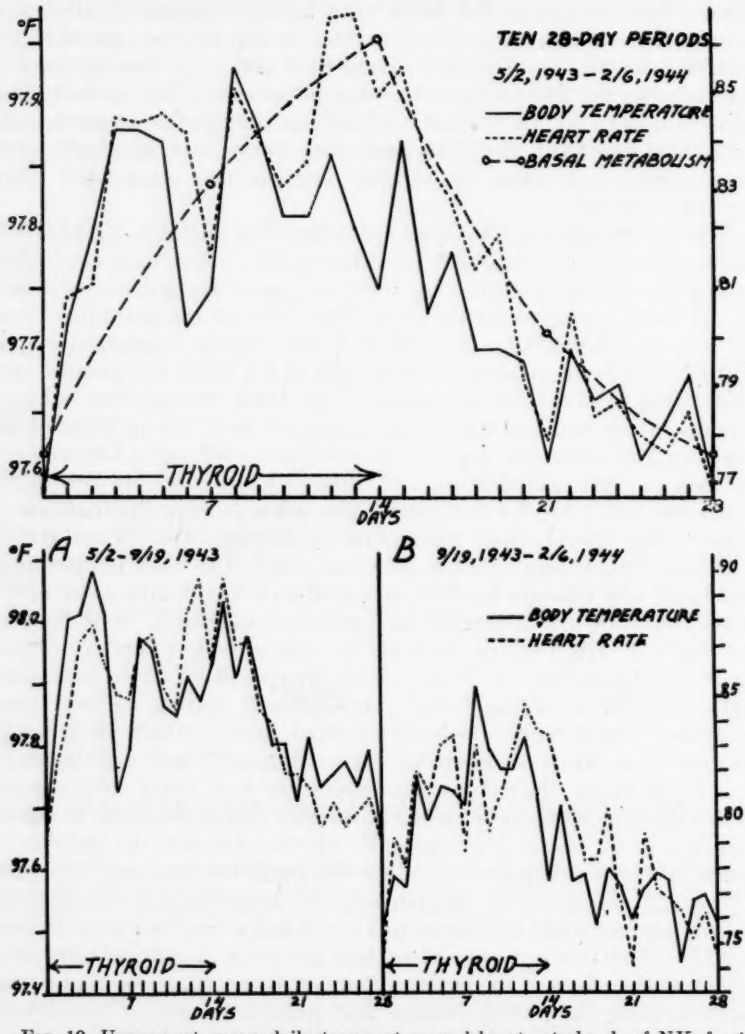

FIG. 10. Upper part, mean daily temperature and heart rate levels of NK, for ten 28-day periods, for the first 14 of which one grain of desiccated thyroid was taken every 12 hours. The mean basal metabolism rates at the end of 7, 14, 21, and 28 days were: 86.2, 89.1, 83.1, and 80.7%, while the corresponding mean body weights were: 151.2, 151.1, 152.6, and 153.6 lbs. Lower part, mean daily levels based on the same data as was upper part, but divided into two 140-day portions (summer and winter.)

while the body weight fell, also mostly during the first week. The reverse occurred during the period of no thyroid, the curve being concave, instead of convex, upward. The body temperatures and heart rates also executed the greater part of their climb during the first week

and showed the sharpest descent during the third. The jaggedness of the curves seems to be related to a weekly periodicity (the body temperature and, to a lesser extent, the heart rate levels for Saturday and Sunday of each week (days 6, 7, 13, 14, 20, 21, 27, and 28) being low, irrespective of thyroid intake). That is perhaps why the basal metabolic rates, determined on successive Sundays, were lower than "normal." The mean day-to-day fluctuations of body tempeature and heart rate levels showed a correlation coefficient of 0.916 ($P<0.01$), and a mean change of 23.4 heart beats per degree F., somewhat greater than reflected in the parallel variations of these two values with changes in outdoors temperature. The seasonal differences, however, are quite marked, as seen from fig. 10 *A* and *B*, in which the summer body temperature and heart rate levels are decidedly higher than in the fall-and-winter ones. The correlation coefficient between the mean body temperature and pulse levels for the 10 successive 28-day periods is 0.947; the second was highest with a mean temperature level of 97.96° and a pulse level of 85.4, and the seventh, lowest, with 97.57° and a pulse of 77.5. For a swing of 0.39° the heart rate change was 7.9 beats, or over 20 beats per degree F. The mean difference between each of the 14 days with, and the corresponding days of the 14 days without, thyroid, were highly significant, amounting to, in the summer to 0.19° and 4.5 heart beats, and in the winter, to 0.25° and 5.1 beats, in each case exceeding 20 beats variation per degree F.

VII. *The effect of motion pictures.* As previously reported (Kleitman, 1945) any degree of excitement tends to raise the body temperature above the customary value for the particular hour. Several of our subjects obtained higher than usual temperature figures while attending motion picture shows in the afternoon or evening. Data collected by HK and RN were numerous enough to be treated statistically. The former, on 55 afternoons, spread over a period of more than two years, had mouth temperatures of 99.00° to 100.15°, with a mean of 99.59°. Temperatures taken at about the same hour on 57 afternoons preceding or following "movie" days, ranged from 97.95° to 99.70°, with a mean of 98.66°, or 0.93° lower. Another way to show the effect of motion pictures is to compare the differences between afternoon and basal temperatures on experimental and control days. The mean rises in temperature on 45 "movie" days was 1.69°, while the corresponding value for 54 control days was 0.73°, the differential being 0.96° in favor of the experimental days. Both the 0.93°, from the former treatment, and 0.96° from the latter, are statistically highly significant. RN attended motion picture shows 29 times in the course of two months, viewing 47 feature films, mostly in the evening. At 8 P.M. her control temperatures varied from 98.40° to 99.40°, with a mean of 98.95°, while at the same hour her "movie" temperatures ranged from 99.00° to 99.65°, their mean being 99.42°. The difference between the two means, 0.47°, was highly significant. In this subject,

also, comparing the differences between the mean basal and 8 P.M. temperatures, the rise on control days was 1.15° and on experimental days 1.68°, giving a mean difference of 0.43°, nearly the same as by direct comparison and statistically even more significant.

DISCUSSION

Both body temperature and heart rate are influenced by a variety of endogenous and exogenous factors, some of which serve to establish a number of periodicities in these physiological variables, while others modify or disrupt periodicities thus established. In general, the temperature and pulse cycles are parallel to each other, but in specific situations they may vary in opposite directions. Although the body temperature changes, especially when expressed in terms of absolute values, are extremely small, they are less labile than heart rates and repeat themselves so well as to yield smooth curves, irrespective of the duration of the particular type of cycle. Yet, in a single cycle, even body temperature changes are not easily predictable. The several frequency distribution curves of the diurnal cycle (figs. 1 & 3) illustrate the great dispersion of the readings for almost any hour of the day and night. Under the best of conditions, these distribution histograms arrange themselves into probability curves, with distinct modes, which usually correspond to the means and medians for the same data.

As has been shown some time ago (Kleitman, 1939), the diurnal body temperature curve is established during infancy, with the trough corresponding to the period of sleep. The shape and location of the crest, however, varies with the individual. In some of the subjects there was a peak in the early afternoon, in others, during the evening, and in still others a remarkable semblance to a plateau characterized the diurnal temperature maximum. But even in the latter more than 60% of the temperature readings were different from the prevailing modes. The occupations of AR and JM (fig. 1 C, D, G) who had early afternoon peaks, were the same as those of HF and JH (1 E, F) whose temperatures varied little during the day: they were all supervisors in a cheese factory and were continually moving about. Nor are the two "plateau" curves alike: that for HF is shallow and symmetrical, the small rise from 6 to 10 A.M. being balanced by an equal fall from 6 to 10 P.M, while JH's curve shows an over twice as great a rise in the morning, and a hardly preceptible fall in the evening.

The heart rate follows the diurnal curve closely, except for the postprandial rise in the former, which after the evening meal may appear to go against the rapidly dropping body temperature. Grollman (1929) also observed a rise in pulse rates after meals, but in his subjects they returned to normal within the hour. Boas and Goldschmidt (1932) recall that Haller knew of the postprandial increase in heart rate. They put this increase as 5 to 15 beats per minute in

one hour, with a return to previous level in 2 to 3 hours. The basal, or getting up, temperatures as well as heart rates, are least variable, from day to day in the same individual, and in one person compared to another. Grollman (1929a, 1930), although finding the cardiac output under basal condition more constant than the heart rate, could detect no seasonal variation in the latter. Likewise, Gustafson and Benedict (1928), in 20 female subjects, found no significant seasonal fluctuations in the basal mouth temperatures.

Within the last few years there grew up a literature on the menstrual cycle in body temperature, although Barton (1940), in her excellent historical review, traces early reports to 1870. Little attention was paid to the heart rate variations during the same cycle. King (1914) did notice a general parallelism between basal body temperatures and heart rates during the menstrual cycle, but Rubenstein, (1937) while reporting typical menstrual temperature curves in 10 subjects, disclaimed any "relation between temperature on the one side and blood pressure or resting pulse on the other." It appears that the monocyclic character of the menstrual temperature cycle and the dicyclic one of heart rate cycle, instead of showing no relationship between the two, can be put to advantage in discovering the exact date of ovulation more accurately than by means of body temperature alone. Whereas the basal body temperature rises on consecutive post-ovulative days, the heart rate shows a rise followed by a drop. Using this double check, we found that the position of the ovulation day in the cycle is far from constant, confirming Barton (1942), but that its range and mean value are definite personal characteristics. This is true, even when the duration of the cycle itself undergoes fluctuations from year to year. In our four subjects, the low points in the menstrual temperature curve occurred, on the average, 16.3, 15.3, 13.8, and 10.8 days before the onset of menstruation.

Like Cullis, Oppenheimer, and Ross-Johnson (1922), we found a parallelism between morning and evening body temperature for the menstrual cycle, and the same applies to the temperature levels, or the mean values of several temperatures taken during the diurnal cycle.

Although the pregnancy and lactation data were obtained on one individual, Barton (1940) and others noted an increased body temperature during pregnancy, and Seward and Seward (1936) found that both body temperature and heart rate were higher in this condition.

Mocquot and Palmer (1940) consider axillary temperatures unsatisfactory and insist on rectal temperatures, and Tompkins (1944) also recommends the latter, but Cullis, Oppenheimer and Ross-Johnson (1922) found complete parallelism between rectal and mouth temperatures during the entire menstrual cycle, and Barton (1940) states that "same cyclic phenomena were manifest in oral,

axillary, rectal, vaginal, and uterine temperatures." Following our suggestion (Kleitman, 1944), Davis (1946) found that mouth temperature curves in 500 cases were completely satisfactory.

The exogenous influence of outdoor temperature on both body temperature and heart rate manifests itself, under the conditions of modern indoor existence, only during the portion of the year when no artificial heating is resorted to. Our figures are in agreement with those of Grollman (1930a) whose subjects' heart rate went up from 58 to 64 when the environmental temperature was raised from 68 to 86. It should be noted that the body temperature and heart rate levels of our subjects, and not the basal values, were higher in the non-heating than during the heating season.

Excitement engendered by attending motion picture shows is sufficient to raise the body temperature, and it is possible that listening to certain types of radio broadcasts or reading stimulating books has the same effect.

Changes in the daily routine of work, meals, rest, and sleep can modify the diurnal body temperature and heart rate curves, but there is evidence of a persistence of long established diurnal cycles. The ease with which the change-over is made varies with the individual.

The relation of body temperature or heart rate to the basal metabolic rate of the individual has been a subject of several investigations. Gustafson and Benedict (1928) state that "metabolism tends to be at a low level in the winter and to rise to a higher level during the spring and summer," while Griffith and others (1929) noted a low metabolic rate in the late summer. Rubenstein (1937) first concluded that basal metabolism was independent of body temperature (although his data showed a good correlation between the two), then published curves (1938) showing a fairly complete parallelism between the body temperature and basal metabolic rate during the menstrual cycle in 15 subjects. Williams (1942) found no evidence of any relationship between basal body temperature and metabolic rate, nor of an effect of thyroid on body temperature. Barnes (1942), on the contrary, decided, after studying over 1000 cases, that "subnormal body temperature is a better index for thyroid therapy than the basal metabolic rate." These discrepancies may be due to the relative constancy of basal temperatures and heart rates. Having made use of the mean daily levels, of both body temperatures and heart rates, we could establish a close relationship between these two variables and the basal metabolic rate. While it is impossible to predict the metabolic rate from body temperature and heart rate data for a given individual, changes in metabolic rate, following thyroid medication, can be followed from day to day by means of body temperature and heart rate levels, and the dose maintained or changed.

Lastly, the relationship of heart rate changes to those of body temperature can be expressed as the increase in the number of heart beats per minute accompanying a rise in body temperature of one

degree F. This value varies from 10 to 20 beats in the ordinary diurnal, weekly, or seasonal changes in body temperature. It may be as high as 30 or 35, when the body temperatures are low, but for fever Lyon (1927) and others report an increase of 9 to 10 beats (and a range of 5 to 15 beats) for each degree F. of fever.

All our data point to an individual characteristic level of body temperature and heart rate, as well as a personal equation covering the establishment, maintenance, fixity or modifiability of the short or long cycles of these physiological variables. In his *Human Frontier* Williams (1946) states that "each human being has a metabolic pattern which differs in some respects from that of all his fellows." Our findings are in agreement with this conception and are offered as a contribution to the physiology of the individual.

SUMMARY

Mouth temperatures and heart rates fall into parallel diurnal curves adjusted to the routine of existence. Basal, or getting up, values are least variable from day to day and from one season to another.

Basal, evening, or mean daily mouth temperatures exhibit a single wave during each menstrual cycle, while the heart rates show two waves. The crossing of the ascending body temperature and the descending heart rate curves permits a more exact determination of the occurrence of ovulation than body temperature data alone.

Both body temperatures and heart rates are higher in pregnancy and lower in lactation than during the menstrual cycle.

During the non-heating season there is a direct relationship between the environmental temperature and both body temperature and heart rate.

Attending motion picture shows raises the body temperature above the level prevailing at the particular time of the day.

Administration of desiccated thyroid raises the body temperature and heart rate in a manner that allows these variables to be used as an index of changes in basal metabolism.

When the body temperature and heart rate vary in a parallel fashion, a rise of one degree F. in the former entails an increase in 10–20 beats per minute in the latter.

ACKNOWLEDGMENTS

We are greatly indebted to the several subjects who went through the painstaking but tedious routine of gathering the reported data by self-observation over long periods of time.

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INFLUENCE OF THE DIET UPON THE NEPHROSCLEROSIS, PERIARTERITIS NODOSA AND CARDIAC LESIONS PRODUCED BY THE "ENDOCRINE KIDNEY"

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EARLIER observations had shown that nephrosclerosis, periarteritis nodosa and myocardiac lesions resembling rheumatic carditis can be produced in rats (especially after unilateral nephrectomy) by continuous exposure to various types of stress or treatment with either desoxycorticosterone or corticotrophic anterior-pituitary preparations. It was noted, however, that these pathologic changes are caused by the above agents only if the animals are kept on diets rich in sodium chloride; conversely, diets poor in NaCl (as well as those resulting in a loss of sodium because of added NH_4Cl) protected our experimental animals against the above-mentioned changes normally produced by stress, desoxycorticosterone or anterior-pituitary preparations.

A high protein content of the food failed to influence the renal and cardiovascular lesions caused by desoxycorticosterone acetate, but aggravated those caused by stress or crude anterior-pituitary extracts. (For literature see: Selye, 1946a; Selye, 1947a; Selye, 1947b.)

More recently we found (Selye and Stone, 1946) that with a special surgical procedure it is possible to reduce the blood pressure in the left kidney of the rat to the level of the colloid-osmotic pressure of the blood. Thus filtration pressure is completely abolished and hence the production of urine ceases. At the same time the kidney transforms itself into a massive epithelial organ whose functions are apparently purely endocrine. Bearers of such "endocrine kidneys" acquire a particularly pronounced, and rapidly fatal, hypertension. At the same time they develop malignant nephrosclerosis in the contralateral (urine-producing) kidney, periarteritis nodosa (especially of the mesenteric and pancreatic vessels) and myocarditic changes, similar to those seen in rheumatic fever. Thus, bearers of an endocrine kidney exhibit lesions essentially similar to those which we had previously produced by stress, anterior-pituitary extracts or desoxycorticosterone.

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Preliminary observations suggested that the morphologic changes in the cardiovascular system and kidney as well as the hypertension produced by the "endocrine kidney" remain uninfluenced by the protein and sodium concentration of the diet (Selye, 1947c; Selye, 1948). This finding was rather unexpected since these same manifestations can be prevented so readily by such dietary measures if the changes are elicited by stress, desoxycorticosterone or crude pituitary extracts.

It will be recalled, furthermore, that a number of earlier investigators claimed that protein (Verney and Vogt, 1938; Handler and Bernheim, 1948) or sodium (Verney and Vogt, 1938; Hantschmann, 1931; Grollman and Harrison, 1945 and Grollman, 1945) poor diets inhibit the development of hypertensive disease in animals subjected to partial constriction of the renal artery or other interventions suitable for the production of renal hypertension. Others, however, obtained inconclusive results (Maclachlan and Taylor, 1940; Philipsborn et al, 1941 and Guerrant et al., 1943). The rice and fruit-juice diet advocated by Kempner (1945) which is poor both in protein and in sodium, also proved to be effective in reducing the blood pressure in animals suffering from experimental renal hypertension (Grollman and Harrison, 1945).

In view of these contradictory data, we decided to re-examine this problem systematically on a large number of animals, using the endocrine kidney technic as a means of producing acute, malignant, renal hypertension. Rats in which endocrine transformation of the kidney has been obtained by our surgical procedure appeared to be singularly well suited for this type of experimentation. Results obtained in the rat are directly comparable with our earlier work concerning the influence of dietary factors upon hypertensive disease caused by stress, desoxycorticosterone or anterior-pituitary extracts in this species. Furthermore, the endocrine kidney technic has the advantage of producing a uniform degree of renal hypertension, since excessive constriction of the artery causes necrosis, while insufficient constriction, which fails to inhibit urine formation, results in hydronephrosis and hence destroys the left kidney (whose ureter is ligated). Either of these experimental errors is readily detectable at autopsy and only bearers of perfect endocrine kidneys were used for the evaluation of our data.

EXPERIMENTAL

In the present series, we employed female albino rats weighing 130–150 gm.; they were killed on the 11th day after the "endocrine kidney operation." The latter was performed according to the technic of Selye and Stone (1946) and Selye (1946c), using the modification in which the ureter of the endocrine (left) kidney is ligated.

All animals were fed a synthetic basic diet in which only the protein (casein) and sodium (NaCl) concentration was varied. Groups

I–VI were placed on a low protein diet, which contained 10% casein and was made up as follows:

Basic Diet

	(gm. per 100 gm. of diet)
Casein	10
Cornstarch	83
Fat (Mazola)	1
Cod liver oil	1
Celluflour	1
Mineral mixture (see below)	4
Supplements (see below)	<1

Groups VI–XII were kept on essentially the same diet but containing 30% casein; correspondingly the carbohydrate (cornstarch) concentration was diminished from 83 to 63%.

In both these diets the mineral mixture was essentially identical with that described by Steenbock and Nelson (1923).

Mineral Mixture

(parts used in making the mixture of which diet contains 4%)

NaCl	23.4
$MgSO_4 \cdot 7H_2O$	24.6
Na_2HPO_4	14.2
K_2HPO_4	69.6
$CaHPO_4 \cdot 2H_2O$	69.8
Ca. Lactate. $5H_2O$	15.4
Ferric Citrate	1.2
KI	.16

The vitamin supplements employed were as follows:

Vitamin Supplements

	(mg. per 100 gm. of diet)
Thiamine chloride	0.8
Riboflavin	0.8
Pyridoxine	0.8
Ca. pantothenate	1.5
Nicotinic acid	1.5
Choline chloride	400.0
Inositol	100.0
p-amino-benzoic acid	30.0

The minerals were simply added to the other dry components of the diet, while the vitamins were first taken up in a little water and then thoroughly mixed with the other dietary constituents, thus sufficiently moistening the dry material to give it the consistency neces-

sary to prepare small food balls, which were placed into the animals' cages.

In groups IV, V, VI, X, XI and XII, excess of sodium was administered by adding 4% NaCl instead of a corresponding amount of corn starch.

In group XIII we wished to examine the effect of simultaneous complete deprivation in Na and Cl on a low-protein diet. Here, the NaCl was eliminated in the mineral mixture and K_2HPO_4 was substituted for Na_2HPO_4. While in all other groups the animals were given tap water to drink, in group XIII only distilled water was allowed in order to eliminate any NaCl intake through the drinking fluid. As a further precaution, the animals of group XIII were placed on the NaCl free ration 10 days before the endocrine kidney operation, to insure that their Na-balance would be negative before the experiment commenced.

EXPERIMENTAL OBSERVATIONS

The results of our observations are summarized in Table I.

The "*survival ratio*" as listed in the fourth column gives the number of animals used for the compilation of these data (all animals bearing imperfect endocrine kidneys were discarded) and (in bold type) the number that survived until the 11th day. It will be seen that all intact controls (Groups I, IV, VII and X) survived. Another type of control consisted in performing the endocrine kidney operation but following this—during the same intervention—by the removal of the left kidney. Controls of this type were deemed necessary in order to eliminate the possible influence of the partial constriction of the aorta, quite apart from any "endocrine transformation" of renal tissue. It will be noted that these controls (Groups III, VI, IX and XII) also survived to the end of the experiment with the sole exception of one animal in group VI. On the other hand, the mortality during the 11 day observation period was very high in all groups in which an endocrine kidney was present (Groups II, V, VIII, XI and XIII). The diet did not appear to exert any very significant influence upon the survival rate of animals possessing an endocrine kidney. Essentially the same is true of the loss of body weight which was manifest in all these groups while the intact controls continued to grow and those in which the endocrine kidney was immediately removed after the operation showed only an insignificant loss of weight, if any.

The weight of the *left kidney* is expressed in mg. as well as in mg./100 cm.^{2} of body surface. The latter method of expression gives perhaps a more correct picture of the amount of kidney tissue available in proportion to the metabolic needs of the organism. It will be noted that the weight of the kidney decreased considerably as a result of the "endocrine transformation" and this involution appears to have

TABLE 1

INFLUENCE OF THE DIET UPON THE MORPHOLOGIC LESIONS INDUCED BY THE "ENDOCRINE KIDNEY"
(Means and Standard Errors)

Group	Treatment	Diet	Survival ratio	Body weight		Left kidney		Right kidney			Heart			Pancreas periarteritis nodosa (in %)	Adrenals (mg.)
				Initial gm.	Final gm.	Mg.	Mg./100 sq. cm. body surface	Mg.	Mg./100 sq. cm. body surface	Nephrosclerosis	Mg.	% of body weight	Myocarditis (in %)		
I	Intact Controls	10% Casein	8:8	130±3	138±4	464 ±19	191± 7	485 ±19	200± 7	0	491 ±16	0.35±0.01	0	0	27±2
II	Endocrine Kidney	10% Casein	22:15	142±2	97±3	248 ±11	131± 7	637 ±20	338±12	48	511 ±18	0.53±0.01	50	70	44±2
III	Endocrine Kidney Removed	10% Casein	15:15	145±2	140±3	—	—	706 ±23	294± 8	0	458 ±17	0.33±0.01	0	0	39±1
IV	Intact Controls	10% Casein 4% NaCl	7:7	130±2	136±3	526 ±24	219± 8	548 ±26	227± 9	0	473 ±20	0.35±0.01	0	0	28±2
V	Endocrine Kidney	10% Casein 4% NaCl	19:8	146±4	98±2	257 ±26	143±14	694 ±51	352±28	57	518 ±34	0.54±0.02	60	48	56±7
VI	Endocrine Kidney Removed	10% Casein 4% NaCl	10:9	145±4	135±4	—	—	702 ±17	295± 6	0	493 ±24	0.36±0.02	0	0	41±3
VII	Intact Controls	30% Casein	8:8	130±2	145±4	579 ±26	231± 8	603 ±29	240± 9	0	529 ±23	0.36±0.01	0	0	33±1
VIII	Endocrine Kidney	30% Casein	27:14	146±2	112±5	271 ±16	129± 6	775 ±33	369±13	52	564 ±21	0.50±0.02	70	63	43±2
IX	Endocrine Kidney Removed	30% Casein	11:11	147±3	147±3	—	—	842 ±25	335± 8	0	481 ±20	0.33±0.01	0	0	45±3
X	Intact Controls	30% Casein 4% NaCl	8:8	130±2	148±3	646 ±18	254±6	670 ±17	266± 5	0	528 ±28	0.35±0.01	0	0	34±1
XI	Endocrine Kidney	30% Casein 4% NaCl	17:8	144±2	122±5	308 ±31	138±13	823 ±21	369± 8	57	586 ±18	0.48±0.02	71	60	46±3
XII	Endocrine Kidney Removed	30% Casein 4% NaCl	10:10	143±3	140±3	—	—	906 ±38	370±14	0	502 ±18	0.36±0.02	0	0	48±2
XIII	Endocrine Kidney	10% Casein Na and Cl free	9:8	131±2	117±6	341 ±15	158± 7	671 ±22	312±17	48	574 ±19	0.50±0.02	52	54	44±3

been entirely independent of the dietary casein and NaCl concentration. On the other hand, among the intact controls, the mean kidney weight was lowest in group I (low casein, low NaCl diet), somewhat higher in group IV (low casein, high NaCl diet), still higher in group VII (high casein, low NaCl diet) and highest in group X (high casein, high NaCl diet). Though not specifically mentioned in the table, all the endocrine kidneys exhibited essentially the same histologic structure, the most characteristic features of which were involution of the glomeruli, loss of eosinophil granules and disappearance of the brush border in the proximal convoluted tubule cells, obliteration of the tubular lumina by proliferating lining epithelium and marked proliferative changes in various parts of the proximal convoluted segment, especially in the distal part, the so-called spiral segment. Nephrosclerosis or hyalinization of blood vessels was never observed in endocrine kidneys, even when the contralateral kidney showed most pronounced changes of this type.

The mean weight of the *right kidney*, among the intact control animals, ran roughly parallel to that of the left. The significance of this kidney-weight increasing effect of dietary protein and NaCl is further supported by the weights of the right kidneys in the animals whose left kidney had been removed at the beginning of the experiment (Groups III, VI, IX and XII). This exhibits essentially the same rise with increasing protein and NaCl concentrations in the diet, the only difference being that here the right kidney is much larger because of the compensatory hypertrophy produced by ablation of the contralateral organ.

The weight of the right kidney in those animals which bear an endocrine kidney on the other side (Groups II, IV, VIII and XI) is less markedly influenced by dietary factors. Histologic examination of these kidneys shows them to be affected by nephrosclerosis with dilatation of tubules, deposition of hyalin material (casts) in their lumina and hyalinization of the blood vessels. Therefore most of the enlargement cannot be regarded as a true compensatory hypertrophy but is more comparable to the increase in renal weight seen in the acute stage of other types of nephrosclerosis, for instance, that produced by desoxycorticosterone or anterior-pituitary preparations.

The nephrosclerosis is listed as a percentage of the theoretic maximum which could have been obtained. The diagnosis was made by one of us upon inspection of histologic slides labelled only with code numbers whose significance he did not know. This was deemed necessary to assure absolute objectivity of this, otherwise rather subjective, type of diagnosis. The degree of nephrosclerosis was expressed in a scale ranging from 0 to+++; hence the theoretic maximum of +++ in all animals was considered as equivalent to 100%. It will be noted that when so expressed, the degree of nephrosclerosis was approximately 50% in all groups including even group XIII in which a casein-poor diet was given in the complete absence of NaCl.

The *heart* weight is expressed both in mg. and as a percentage of

body weight. It will be noted that according to either manner of expression, the heart weight is increased in the endocrine kidney bearing rats (Groups II, V, VIII, XI, XIII) as compared to the corresponding intact controls or those in which the endocrine kidney was removed. The degree of this cardiac hypertrophy was not significantly influenced by the diet. The heart weight is a fairly accurate expression of the mean blood pressure during the entire experimental period. Indeed some investigators believe the heart weight to be a more accurate index of hypertension than the occasional actual measurement of the blood pressure at intervals throughout the experiment, because it is less likely to be influenced by sudden transient variations. No systematic blood-pressure measurements were made in groups I–XII of this series, yet careful determinations of this type (performed under light ether or nembutal anesthesia by the direct method) revealed that in group XIII the endocrine kidney caused marked hypertension (up to 180 mm. of mercury mean pressure in some cases). This shows that renal-pressor material can be produced, and when present can raise the blood pressure in the complete absence of NaCl even on low-protein diets.

The myocarditic lesions produced by the endocrine kidney in groups II, V, VIII, XI and XIII were likewise determined as a percentage of the maximum total, on the basis of histologic examination under conditions identical with those described above for the kidney. It will be noted that the diet also failed to influence the intensity of the histologically demonstrable cardiac damage caused by the endocrine kidney.

Periarteritis nodosa was noted on histologic sections in the pancreas since the vessels of this organ are especially subject to periarteritic lesions. The diet did not appear to influence the intensity of the hyalinization and granuloma formation.

The *adrenals* were greatly enlarged in all endocrine-kidney bearing rats, again without any manifest relationship to the diet consumed. It will be recalled that the corticotrophic effect of stress and of impure anterior-pituitary extracts is greatly dependent upon the dietary protein intake although independent of the sodium content of the food (Selye, 1947b). It has been assumed in previous publications from our laboratory that a certain amount of protein catabolism is essential for the maximal production of adreno-corticotrophic hormone; perhaps in the present experimental series the protein-catabolic effect of the renal pressor material was sufficient to furnish optimal amounts of protein catabolites, even when dietary protein intake was low.

DISCUSSION

Our earlier observations had shown that ingestion of excessive amounts of NaCl increases, while sodium deprivation (due to a diminished Na intake or a forced Na-loss through the urine, e.g., after NH_4Cl medication) diminishes the hypertensive and nephrosclerotic activity of desoxycorticosterone (Selye and Stone, 1943; Selye and

Hall, 1943a; Selye and Hall, 1943b; Selye et al., 1944). It has also been demonstrated that the production of nephrosclerosis, vascular changes and hypertension by anterior-pituitary preparations is likewise inhibited by sodium depletion (Hall et al. 1945). The adrenotrophic effect of crude anterior-pituitary extracts, on the other hand, remains uninfluenced by sodium but is greatly diminished in animals maintained on low-protein diets. At the same time protein depletion also prevents the production of nephrosclerosis, hypertension and cardiovascular lesions by anterior-pituitary extracts (Berman et al., 1945; Selye, 1946b; Hay et al., in press). On the other hand the nephrosclerotic and hypertensive actions of desoxycorticosterone remain uninfluenced by dietary protein (Prado et al., 1947).

From all these observations it appears that the corticotrophic activity of crude anterior-pituitary extracts (and probably also the corticotrophin production under the influence of stress) are dependent upon the protein intake but independent of the sodium intake; conversely, the action of corticoids such as desoxycorticosterone is dependent upon the availability of sodium but independent of protein.

The present experimental series clearly shows that the hypertension as well as the accompanying cardivascular and renal changes produced by the endocrine kidney are independent of both the protein and the sodium content of the diet.

As mentioned in the introduction to this paper, pertinent observations of earlier investigators—who studied the effect of the protein and sodium concentration in the diet upon the development of renal hypertension—were rather inconclusive. It must be kept in mind that none of these workers observed animals in which the "endocrine transformation" of the kidney was as complete as in our rats. We cannot give an explanation for the apparently positive results of some investigators but feel that the data presented in this publication clearly indicate that, under the experimental conditions used by us, renal hypertension develops irrespective of the dietary protein and sodium. It will be recalled that the "endocrine kidney technic" suddenly causes complete endocrine transformation of the entire renal parenchyme; it is possible that in the face of such sudden maximal transformation, dietary constituents cannot further increase the production of renal pressor substances. Yet, in the event of less drastic surgical interventions, nutritional factors may play a rôle, perhaps by increasing the production of hypophyseal and corticoid hormones or by augmenting the "endocrine transformation" of individual nephrons under the influence of corticoids.

SUMMARY

Experiments on the rat indicate that the production—by the "endocrine kidney" technic— of hypertension and its morphologic accompaniments (nephrosclerosis, cardiac hypertrophy, myocarditis,

periarteritis nodosa) remain uninfluenced by the protein and NaCl concentration of the diet.

These findings are contrasted with the marked dependence upon sodium of the production of these same lesions by desoxycorticosterone or anterior-pituitary extracts.

High protein diets do not influence the production of hypertensive disease by desoxycorticosterone but they markedly increase this same effect of crude anterior-pituitary extracts.

It is concluded that protein increases the corticotrophic action and sodium augments the effect of mineralo-corticoids. Anterior-pituitary hormones stimulate corticoid production, while the corticoids cause nephrosclerosis and the formation of "endocrine nephrons." The diet influences the "endocrine transformation" of kidney tissue under the influence of such hormonal stimuli but—as shown by the present experimental series—the secretion and action of the renal pressor hormones themselves remain uninfluenced by the sodium and protein intake.

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ADRENAL AUTOTRANSPLANTS WITH HEPATIC PORTAL DRAINAGE IN THE RAT

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IT HAS been known for many years that the liver can inactivate estrogenic hormone or impair the function of ovarian tissue. It has, likewise, been found that epinephrine is destroyed in the liver since injections of it into the splenic vein produce about a fourth the rise in blood pressure that can normally be expected (Philpot and Cantoni, 1941).

Progesterone, on the other hand, is not inactivated by liver pulp "in vitro" (Engel, 1944). There are also indications that the function of cortical tissue of the adrenal is not impaired by the liver since Eversole, Edelmann and Gaunt (1940) succeeded in maintaining the lives of two of ten rats with adrenal autografts in the liver. Failure in eight of their experiments may have been a matter of technique or the liver may have been antagonistic toward hypertrophy of the tissue of the adrenal.

More recently Vogt (1943) has claimed that the body disposes of released cortical hormone of the mammalian suprarenal at great speed since no detectable amount of the hormone was in the blood from the right side of the heart or from the femoral artery in the dog and cat. The result was the same in the eviscerated animal and, according to him, if the liver played a predominant role in the inactivation of the hormone, the exclusion of the liver from the circulation would lead to the accumulation of cortical substance in the general circulation. No hormone was found in the right atrium and femoral artery by Vogt and, therefore, little existed to be inactivated by the liver. Since the cortical substance encountered few tissues in going from the adrenal to the right atrium and these tissues disposed of most of the hormone the liver might be a great inactivator of cortical substance if quantities of the hormone reached this organ.

The purpose of this investigation has been to learn: (1) if adrenal transplants in the liver or spleen will maintain the life of the rat as well as autografts in the kidney; (2) how much of the adrenal cortex must be transplanted for survival of the animal after bilateral adrenalectomy.

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METHODS

In these experiments rats of the Long-Evans and Wistar strains were used. Since hair growth in the rat is accelerated in the absence of cortical material (Butcher, 1937) (Ralli and Graef, 1943), normal hair growth and the survival of animals were used as indicators of a sufficient supply of cortical hormone in the body. Operations were made soon after weaning or on the twenty-second day of life.

Both adrenals were removed via the dorsal approach and then either an entire adrenal or a portion of one adrenal was autotransplanted into the liver, spleen or kidney. A portion of an adrenal was autotransplanted to compare the inactivating power of the tissues of the kidney and of the liver on the cortical hormone. If the inactivating power of the liver was greater than that of the kidney, more cortical materials would have to be present to maintain life. When a half or a fourth of an adrenal was used as a transplant, an adrenal was cut as nearly as possible into such fractions under the stereoscopic microscope. Transplants were made easily under the capsule of the kidney as described in a previous paper (Butcher, 1937). For holding an autograft of an entire adrenal to the spleen, a fine thread was run through the capsule of the adrenal and then the thread was tied around the spleen. Autografts were anchored and retained with difficulty in small slits in the liver and spleen.

One per cent salt solution served as drinking water for 4 days after the operation and thereafter, only tap water was available. Feed consisted entirely of Purina dog chow.

Animals were sacrificed at intervals following the operations. The autografts were recovered; fixed in Bouin's; stained in hematoxylin and eosin; and then studied. When the animals were sacrificed, the regions around the kidneys were carefully surveyed for accessory cortical material and this tissue was fixed and studied histologically. Animals possessing accessory cortical bodies were not included in this study. Autografts were also recovered as often as possible from animals dying of cortical insufficiency.

AUTOGRAFTS OF ONE-FOURTH OF AN ADRENAL

Twelve of 16 animals receiving autografts of one-fourth of an adrenal in the liver died within 14 days after the operation. On histological examination, the grafts of these animals were found isolated by a thick capsule. The transplants often contained small islands of regenerated tissue immediately internal to the capsule and necrotic masses of cellular debris and pigmented degenerating cells were found centrally. The small islands which apparently originated from the capsule were incapable of supporting the animal.

The other four animals were sacrificed at 35, 70, 100 and 140 days after the operation. One had a litter of six when the graft was 56 days old and the puppies were guarded with great care. They all died, however, within 10 days because of the inadequate milk supply of the mother. Histological examination of the adrenal grafts of these four sacrificed rats showed that they were surrounded with a thin capsule. The grafts were large and were composed of a very vascular glomerulosa and fasciculata but no reticularis was present.

Animals with autografts of one-fourth of an adrenal in the kidney survived no better than those with similar autografts in the liver. Six of the eight animals receiving such grafts died within 12 days after the transplantation. The autografts which were recovered from them were composed of areas of regenerated cortical cells beneath a thick capsule. Two other animals with one-fourth autografts gained weight and were sacrificed 35 and 70 days after the operation. The large grafts from these two animals had a thin capsule and most of the cells were glomerular in character.

Hair growth was accelerated in practically all animals which received an autograft of one-fourth of an adrenal and which died within 12 days after the transplantation.

AUTOGRAFTS OF ONE-HALF OF AN ADRENAL

Four of the ten animals receiving one-half of an adrenal in the liver died within 12 days after the operation. Regenerated islands of cortical tissue were often found next to the capsule in the grafts recovered from the dead animals. The bulk of the transplants, however, consisted of pigmented degenerating cells.

The six other animals receiving one-half adrenal in the liver survived and were sacrificed 48, 70, 96 and 102 days after the transplantation. The recovered grafts were very large and were surrounded by a thin capsule which was continuous with septa extending throughout the gland. Most of the graft consisted of glomerulosa and fasciculata regions. In some areas which were not always centrally located the cells were arranged in a reticular pattern. One rat had a litter 80 days after the operation but the puppies died soon after birth.

Autografts of one-half adrenal in the kidney were equally as successful as in the liver. One animal died on the fifteenth day and two others on the eighteenth day after the operation. The death of one animal 26 days following grafting was surprising since the recovered graft showed several large regenerated islands. These islands were composed of closely packed cells and were separated by septa. They had not reached the differentiation in zonation attained by most grafts in surviving animals. Other animals gained weight and were sacrificed 37, 75 and 140 days after grafting. Nine of this group of 13 animals with grafts in the kidney survived longer than 30 days.

Many of the rats receiving one-half of an adrenal as a graft showed accelerated hair growth. The animals surviving longer than 30 days were quite inactive and failed to grow as rapidly as litter mates.

AUTOGRAFTS OF AN ENTIRE ADRENAL

In most of the first operations where an entire adrenal was transplanted into the spleen, the animal's life was not maintained longer than 12 days. When the transplants were recovered from these rats, they were found to be degenerate necrotic masses and to contain no

surviving tissue other than remnants of a capsule. The capsule of the gland was left intact at the time of the operation. This probably prevented rapid revascularization and thus complete degeneration followed. When the capsule was punctured or incised and the gland was transplanted, most of the animals survived. Considerable difficulty was experienced, however, in getting such a large piece anchored in the spleen. A better method was then devised which consisted of running a fine thread through the capsule and tying the thread around the spleen. This thread served to anchor the graft in a slit previously made in the spleen and also provided a path for rapid ingrowth of blood vessels. Twelve of 14 animals survived longer than 30 days with such technique. At intervals (44 to 180 days) following the operation the recovered grafts in many instances consisted of very large regenerated masses partly within the spleen and partly on its periphery. Glomerulosa and fasciculata zones were identifiable and the caspule was usually thin. In other instances, the autograft was quite similar in structure to the normal adrenal: all zones were represented and a region filled with blood was present in the center. No medullary material had survived. One rat bore a litter of seven 68 days after the autografting and another rat had a litter of eight 82 days after the transplanting. All the puppies died in one litter and only two of the other litter survived until weaning time. Autografts in the spleen which had maintained life for 40 days were removed from three animals. Death followed on the 6th, 8th and 14th day after removal.

Entire adrenals were difficult to anchor in the liver. A few animals died within 12 days following the transplanting. Grafts when recovered had a thick capsule and a few small islands around the periphery. These islands apparently were not enough to maintain the life of the animal. Most of the animals (12 of 17) which received an entire adrenal survived longer than 30 days. When sacrificed, the autografts were surrounded by a thin capsule and were composed of large compact masses of cells showing no arrangement.

The rats receiving an entire adrenal as an autograft were more active and had better health than those receiving a transplant of a portion of an adrenal. The autografts recovered from animals receiving an entire adrenal as a transplant, while no greater in mass, were better differentiated and the zonation was usually more pronounced than the autograft resulting from a portion of an adrenal.

DISCUSSION

Following bilateral adrenalectomy, adrenal autotransplants in the liver and spleen, although more difficult to anchor, have maintained the lives of hooded and white rats equally as well as autotransplants in the kidney.

When an entire adrenal was tied to the spleen, about 85% of the animals survived longer than 30 days. An entire adrenal was anchored and retained with difficulty in the liver and only 71% of the animals

lived longer than 30 days. Sixty percent survival for more than 30 days resulted after the autografting of one-half of an adrenal in the liver and 69% survival in the case of similar grafts in the kidney. Only 25% survived longer than 30 days with an autograft of one-fourth of an adrenal in either the kidney or the liver. Death often occurred in animals with one-fourth of an adrenal five or six days after the operation while death in animals with an entire adrenal was more common 11 or 12 days after the operation. These experiments showed that the inactivating power of the liver on the cortical hormone was not greater than that of the kidney.

The degenerative and regenerative changes in the adrenal autografts have been followed rather carefully. In general my observations confirm those made by Ingle and Higgins (1938). These authors claim that necrosis begins in the center of the entire organ, extends peripherally, and in 48 hours most of the gland except the capsule is destroyed. In my preparations the moribund cortical cells disappear in a peripheral to central sequence as the polymorphonuclear leucocytes, lymphocytes and macrophages invade them.

Areas of regenerated cortical cells begin to appear beneath the thick capsule within three or four days after the operation and by seven or eight days there are often large islands of cortical tissue. These areas enlarge, usually coalesce, but in many instances remain separated by septa to form an irregular shaped graft. The inequality in the regenerative capacity of the regions of the capsule is difficult to explain as Williams (1947) has pointed out. In the case of a portion of the gland as the autograft, the peripheral capsule immediately surrounds the more centrally located cells which undergo degeneration. As regeneration progresses, islands of newly formed cortical cells appear under the capsule and the capsule usually becomes thinner.

Failure of autografts of one-fourth of an adrenal or one-half of an adrenal to maintain life as well as entire adrenal autografts may have been due to insufficiency of cortical hormone in the implants to carry the animal over the period until the grafts regenerated or to the amount of capsule available for regeneration or to both. Since the gland with the exception of the capsule necroses rapidly after the transplantation, the amount of capsule available for regeneration is probably the determining factor. Hair growth was frequently accelerated in animals receiving one-fourth or one-half of an adrenal indicating cortical insufficiency.

Normal zonation seldom occurred in any of the grafts and for the most part the transplants consisted of cells with a glomerulosa and fasciculata arrangement. The grafts enlarged with time but a reticular zone was as common in 40 day old grafts as it was in much older grafts.

The size reached by the graft has been discussed and investigated at great length by Ingle and Higgins (1938) and Wyman and tum

Suden (1937). The former conclude that the size can . . . "be explained by the assumption that the adrenotropic activity of the pituitary body is responsive to the physiologic requirements for cortin." Several of my rats with autografts had litters yet with apparently normal maternal care they lost most of the litter due to their inability to supply adequate milk. In this instance an adjustment was not made between the physiologic requirements for cortin and the adrenotropic activity of the pituitary body.

SUMMARY

Following bilateral adrenalectomy, either an entire adrenal or a portion of an adrenal was autotransplanted into the liver, the spleen or the kidney of the rat. The amount of the gland transplanted and the percentage of animals surviving for more than 30 days has been as follows: entire in spleen 85%; entire in liver—71%; one-half in kidney—69%; one-half in liver—60%; one-fourth in kidney—25%; and one-fourth in liver—25%.

In surviving transplants, regions of the capsule produced new areas of cells which often remained separated by septa. Normal zonation seldom occurred. Regeneration was equally as good in the liver and spleen as in the kidney.

The tissues of the liver and spleen appear to have no more inactivating effect than the tissues of the kidney on the cortical hormone.

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EFFECTS OF THIOURACIL FEEDING ON RESISTANCE TO LOW ENVIRONMENTAL TEMPERATURE[1]

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In 1943 Leblond and Gross observed that thyroidectomized rats failed to survive at low environmental temperatures unless administered thyroid hormone. Since thiouracil inhibits thyroxin synthesis in the body, impaired survival at low environmental temperatures might also be expected following prolonged administration of this drug. In the present experiment adult female rate were made hypothyroid by thiouracil feeding, and their length of survival determined under cold room conditions with and without thyroxin administration.

PROCEDURE AND RESULTS

Forty-seven female rats of the Sprague-Dawley strain were raised to maturity on a stock ration and selected for the present experiment at approximately 10 weeks of age and an average body weight of 170.1 g. (range 148 to 192 g.). Forty-one rats (group I) were fed a purified ration consisting of thiouracil 0.3%, casein[2] 22.0%, salt mixture[3] 4.5%, whole liver powder[4] 10.0%, cottonseed oil (Wesson) 10.0% and sucrose 53.2%. To each kg. of the above diet were added the following synthetic vitamins: thiamin hydrochloride 72 mgms., riboflavin 9 mgms., pyridoxine hydrochloride 15 mgms., calcium pantothenate 67.2 mgms., nicotinic acid 60 mgms., 2 methyl-naphthoquinone 5 mgms. and choline chloride 1.2 g. Each rat also received once weekly a vitamin A-D concentrate[5] containing 150 U.S.P. units of vitamin A and 15 U. S. P. units of vitamin D together with 3 mgms. of alpha-tocopherol acetate. Six rats (group II) were fed a diet identical with the above but with thiouracil omitted and replaced by an

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[2] Vitamin Test Casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

[3] Salt Mixture No. 1 (Sure, 1941).

[4] Whole Dried Liver Lowder, Armour and Co., Chicago, Ill.

[5] Nopco Fish Oil Concentrate, assaying 800,000 U.S.P. units of vitamin A and 80,000 U.S.P. units of vitamin D per gram.

equal amount of sucrose. Animals were placed in metal cages with raised screen bottoms to prevent access to feces and were fed the above diets *ad lib.* for 4 weeks.

After 28 days of feeding basal metabolic rates were determined for 9 rats selected at random from group I and the 6 animals of group II.[6] The apparatus used was a closed circuit type with a capacity of 2 liters (Mason and Winzler, 1948). Carbon dioxide was absorbed with sodium hydroxide, and oxygen consumption was determined from pressure changes recorded by means of a water manometer. The respiration chambers were kept at 28°C. and readings obtained were corrected to S.T.P. Food was removed from the animals' cages the evening prior to the metabolism test. At least 6 successive 5-minute intervals were recorded for each animal, with care being taken to record oxygen consumption when animal activity was at a minimum. Following the B.M.R. determinations the above rats were sacrificed and thyroid weights determined. Results are summarized in table 1.

TABLE 1. EFFECTS OF THIOURACIL FEEDING ON OXYGEN CONSUMPTION AND THYROID WEIGHT

Group	Number of animals	Thiouracil content of ration	Body wt.	Oxygen consumption cc/hr/100 g. body wt.[1]	Change in B.M.R.[2]	Thyroid wt.[1]	Thyroid wt. mg./100 g. body wt.
		per cent	g.		per cent	mgms.	
I	9	0.3	174.4	83.3±2.7	−27.9	56.3±6.8	32.3
II	6	0.0	175.6	115.5±6.7	—	10.2±0.5	5.8

[1] Including standard error of the mean.
[2] Compared to the control B.M.R. of Group II.

Findings indicate that 4 weeks of thiouracil administration resulted in a marked increase in thyroid size and a significant decrease in basal metabolic rate, findings indicative of a hypothyroid state. Surviving rats of group I were then divided into 6 groups and were placed in individual metal cages in a large walk-in refrigerator maintained at a temperature of 2±1.5° C. No change was made in the experimental diet but each rat received daily intraperitoneal injections of the following materials: group A, 1 cc. saline solution; group B, 0.5 cc. adrenal cortex extract;[7] group C, 2.5 μg dl-thyroxin;[8] group D, 5.0 μg dl-thyroxin; group E, 10.0 μg dl-thyroxin; and group F, 20.0 μg

[6] We are indebted to Miss Betty Tukich and Dr. R. J. Winzler of the Department of Biochemistry and Nutrition, University of Southern California, for the B.M.R. determinations.

[7] Adrenal Cortex Extract, Wilson Laboratories, Chicago, Ill. The material was administered twice daily in doses of 0.25 cc.

[8] Thyroxin (Synthetic Cryst.), Roche-Organon, Inc., Nutley, N. J. The material was dissolved in .1 N NaOH, adjusted to a pH of 8.0 and diluted to a volume containing 10 μg thyroxin per cc.

dl-thyroxin. Injections were begun the day preceding transfer to the cold room. Treatment was continued for 28 days or until death. Food consumption was determined daily for each rat during the first week of treatment. On the 28th day surviving animals were autopsied and thyroid weights determined. Results are summarized in table 2.

TABLE 2. EFFECTS OF dl-THYROXIN ON SURVIVAL TIME AND BODY AND THYROID WEIGHT OF THIOURACIL-FED RATS UNDER COLD ROOM CONDITIONS

Group	Daily treatment received	Number of animals	Initial body weight	Per cent surviving[1]	Average survival time[2,3]	Gain in body wt. during expt.[3]	Thyroid wt.[3]	Thyroid wt. mgms./100 g. body wt.
			g.		days	g.	mgms.	
A	1 cc. saline solution	6	187.8	0.0	5.7±0.6	—	—	—
B	0.5 cc. adrenal cortical extract	6	187.2	0.0	4.3±0.5	—	—	—
C	2.5 cc. dl-thyroxin	5	188.2	100.0	28.0±0.0	13.4±1.9	79.6±5.8	39.4
D	5.0 μg dl-thyroxin	5	188.8	100.0	28.0±0.0	9.2±4.0	43.6±6.8	22.0
E	10.0 μg dl-thyroxin	5	188.6	100.0	28.0±0.0	22.2±4.2	23.0±2.0	10.9
F	20.0 μg dl-thyroxin	5	188.6	100.0	28.0±0.0	19.0±3.0	22.4±1.6	10.7

[1] Experimental period—28 days.
[2] Averages were computed on the basis of a 28 day survival time for animals alive at the termination of the experiment.
[3] Including standard error of the mean.

Findings indicate that thiouracil-fed rats fail to survive under cold room conditions in the absence of thyroid hormone.[9] Under conditions of the present experiment thiouracil-fed rats in group A survived an average of 5.7 days (range 3 to 7 days). Parenteral administration of adrenal cortical extract was without significant effect on length of survival. Dl-thyroxin, however, at a daily dose as small as 2.5 μg per day was completely effective in maintaining survival during an experimental period of 28 days. All rats administered thyroxin survived and gained weight during the experiment, but differences in growth were not statistically significant. Marked differences in thyroid weight were observed, however, among the 4 groups receiving thyroxin. Thyroid weights were largest for the group administered 2.5 μg thyroxin daily and least for those receiving the 10 or 20 μg levels. At the latter dosage (groups E and F) thyroid weights were significantly less than those observed in similar animals (group I) autopsied prior to the cold room exposure (table 1). The cause of death in groups A and B was not inanition for the food consumption of animals in these groups did not differ significantly in amount from that of animals receiving thyroxin.

[9] Animals corresponding to these in age and weight and fed a similar diet but with thiouracil omitted survived under cold room conditions without thyroxin administration. The average change in body weight of 6 rats over a 28 day period was −2.2 g. Thyroid weight averaged 11.5 mgms. (range 10.0–13.8 mgms.) or 6.2 mgms. per 100 g. body weight.

DISCUSSION

Findings indicate that drug therapy may affect resistance to low environmental temperature. In the present experiment thiouracil feeding was shown to impair survival at low environmental temperatures. Unpublished work from this laboratory indicates that similar results can be obtained by feeding promin (sodium p,p′-diaminodiphenylsulfone N,N′-didextrose sulfonate) recently shown by this laboratory to be goitrogenic (Ershoff, 1948). For both drugs thyroxin will significantly prolong survival at low environmental temperatures. Presumably similar results may be obtained with other antithyroid agents such as rape seed, promizole, thiocyanates, sulfonamides and others (Charipper and Gordon, 1947). Failure of survival at low environmental temperatures appears under these conditions to reflect a deficiency of thyroid hormone. The amount of thyroxin necessary to maintain survival, however, appears to be considerably less than the amounts normally secreted under cold room conditions (Dempsey and Astwood, 1943).

SUMMARY

Rats made hypothyroid by thiouracil feeding failed to survive on exposure to a low environmental temperature. Length of survival under these conditions averaged 5.7 days (range 3 to 7 days). One hundred per cent survival over a 28 day period was obtained in animals similarly treated but administered thyroxin.

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COMPARATIVE PARENTERAL THYROXINE-LIKE ACTIVITY OF NATURAL AND SYNTHETIC THYROPROTEINS STUDIED WITH THE GOITER PREVENTION METHOD[1]

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LOS ANGELES

INTRODUCTION

MANY investigators have observed that the activity of natural thyroid proteins is greater than an equivalent amount of thyroxine.[2] The large disagreement in relative oral potencies between thyroid preparations and thyroxine has been explained on the basis of poor absorption of the relatively insoluble thyroxine comparison standard. The smaller but significant differences in parenteral activity have led some workers to attempt to correlate activity with the total iodine content of natural thyroid proteins (Gaddum, 1929–30; Means, Lerman and Salter, 1933; Lerman and Salter, 1934; Salter, 1935). Others have claimed agreement with the thyroxine content when it is assumed that the L-thyroxine contained possesses twice the activity of DL-thyroxine (Palmer and Leland, 1935; Foster et al., 1936, Parkes, 1947). Still others have reported that thyroxine-like activity could not be correlated with either total iodine content or thyroxine content of natural thyroid proteins (Meyer and Wertz, 1939). It has been difficult to settle the problem because of the large experimental errors involved in the biological methods available for the determination of thyroxine-like activity (e. g. basal metabolic rates, guinea pig weight loss, protection against acetonitrile poisoning in mice, amphibian metamorphosis, etc.).

Dempsey and Astwood (1943) were the first to use the ability of thyroxine to depress the thyroid weights of thiouracil-fed rats as a method of determining thyroid secretion rates under various environmental conditions. Mixner, Reineke and Turner (1944) studied the effect of thiouracil and thiourea on the thyroid gland of the chick, and

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[2] This subject has been reviewed at length in a number of texts including those of Kendall (1929), Harrington (1933), Means (1937), Elmer (1938), Salter (1940).

many of the potential variables in the application of the goiter prevention technique for the biological assay of thyroxine-like activity of thyroidal materials were investigated. These same workers subsequently found that thyroid assays in rats by this technique were directly comparable with results obtained by the standard metabolic method (Reineke, Mixner, and Turner, 1945). Reineke and Turner (1945a) employed this method along with other methods to determine the relative thyroidal potency of L-thyroxine and DL-thyroxine, obtaining essentially the same results with all the different techniques used. This method seemed to be the one of choice and convenience for the biological assay of the thyroxine-like activity of thyroactive substances.

The improvements in the preparation of synthetic thyroproteins by Reineke, Turner and coworkers have been reviewed by Reineke (1946). Reineke et al. (1945) reported agreement between the results of biological assay based on the measurement of basal metabolic rates of guinea pigs, and a modification of the method of Blau (1933, 1935) for the chemical estimation of the thyroxine content of thyroactive iodinated proteins.

This work has been undertaken in order to determine whether the biological activity of natural thyroid corresponds to the thyroxine content of the preparation, and to compare the biological effects of natural and synthetic thyroproteins.

METHODS

Groups of five or six female albino rats of the University of Southern California strain, weighing between 125 and 200 grams, were used throughout these experiments. Prior to the experimental period of 15 days, the animals were fed the stock diet previously employed. The rats were fed ad libitum with stock diet containing 0.3% thiouracil and for 14 days were given daily intraperitoneal or subcutaneous injections of DL-thyroxine or appropriate suspensions or solutions of the natural or synthetic thyroid proteins (the injection volume usually being about 0.20 ml./100 g. body weight, and the thyroxine level in the range of 1 to 10γ/100 g. body weight). On the fifteenth day the animals were weighed and sacrificed, and the thyroid glands removed and weighed to the nearest 0.1 mg. The room temperature was maintained at $75 \pm 3°$ F., and DL-thyroxine controls were used in each series of experiments. Figure 1 shows a typical DL-thyroxine standard curve obtained in Experiment I, reported in Table 2. From Figure 1, using the method of Dempsey and Astwood (1943), the daily secretion rate of 100 to 200 gram female rats of the University of Southern California strain at 24° C. is seen to be 6.0γ DL-thyroxine per 100 gram body weight, which is in the same range as the 5.0γ value reported by Astwood and Bissel (1944) and the 4.1γ DL-thyroxine reported by Monroe and Turner (1946) under somewhat similar environmental conditions. In each experiment, the biological activity of the thyroactive protein was determined from such a DL-thyroxine-thyroid weight curve run simultaneously with the experimental groups. Tables 2 and 3 include no values which give thyroid weights significantly below that of the normal untreated rat.

MATERIALS

The natural thyroid proteins (D-1, D-2, A, B)[3] were analyzed for total iodine by the method of Waters and Beal (1945) and for thyroxine by Blau's (1933) method. Preparations D-1 and D-2 were two samples of USP desiccated thyroid. A and B were commercially obtained, partially purified thyroid preparations. The synthetic thyroproteins (STP) were prepared essentially by the method of Reineke and Turner (1945b). Particular care

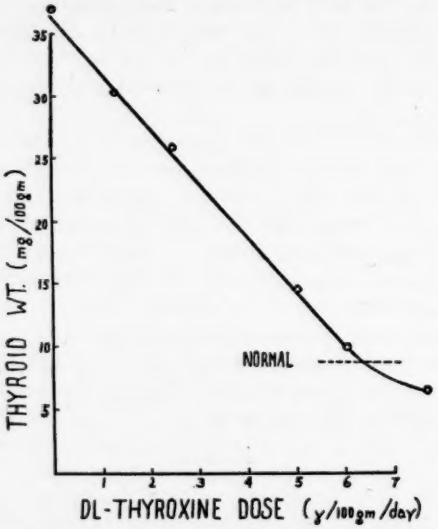

FIG. 1. Typical standard curve showing the effect of DL-thyroxine dose on the thyroid weight in thiouracil-fed rats. Groups of approximately six 150 to 200 gram female rats were fed a 0.3% thiouracil diet for 15 days during which time they received daily intraperitoneal injections of the indicated amount of DL-thyroxine.

was taken to minimize any racemization of L-thyroxine by using sodium bicarbonate instead of sodium hydroxide to dissolve the proteins in all steps of the preparation or testing procedures. STP-1 was subjected to analysis for total iodine and thyroxine. Total iodine was determined again by the method of Waters and Beal (1945). The thyroxine content was determined by the procedure of Reineke et al. (1945) employing a 40% barium hydroxide hydrolysis, and also by Blau's method using a 2 N sodium hydroxide hydrolysis. The average of four individual thyroxine determinations by the former technique was 2.6%. Duplicate analyses on STP-1 by Blau's method gave also 2.6%. Thus, in this limited investigation, there was no evidence of significant differences between Blau's method and that of Reineke et al. for the determination of thyroxine in iodinated proteins. However, the possibility exists that the observed thyroxine values may be high due to the presence of non-thyroxine iodine in the final butyl alcohol fraction. This possibility is strengthened by the biological data reported later. STP-2, 3, 4 were ana-

[3] The natural thyroid proteins were provided by the Harrower Laboratory, Inc. Glendale, California.

lyzed for thyroxine by Blau's method in view of the results obtained with STP-1. IC-1 was an iodinated casein product prepared in such a manner that a minimum amount of thyroxine would be formed. Though it contained 4.1% iodine, its thyroxine content was less than 0.1%. The important analytical data on the various proteins employed in this investigation are compiled in Table I.

The proteins were dissolved or suspended in an excess of dilute sodium hydroxide or 1% sodium bicarbonate with the aid of a mortar and pestle, the pH adjusted to 8.0 ± 0.5 and the solution made up to volume. A highly satisfactory suspension of the natural thyroid proteins was obtained in this manner. The synthetic thyroproteins were readily soluble under the above

TABLE 1. TOTAL IODINE AND THYROXINE CONTENTS OF NATURAL AND SYNTHETIC THYROID PRODUCTS

Preparation	Per Cent Total Iodine	Per Cent Thyroxine
Desiccated Thyroid-1	0.52	0.26
Desiccated Thyroid-2	0.64	0.31
Product A	0.99	0.46
Product B	0.27	0.13
STP-1	10.7	2.6
STP-2	7.7	2.4
STP-3	8.6	2.9
STP-4	8.9	2.3
IC-1	4.1	0.1

conditions. DL-thyroxine[4] was dissolved in a minimum amount of 0.1 N NaOH, the pH ajdusted to 8.0 ± 0.5 and the solution made up to volume.

RESULTS

Thyroxine-like activity of parenterally administered natural thyroid proteins. Table 2 lists the data obtained on three independent experiments testing the thyroxine-like activity of the various thyroactive proteins when administered intraperitoneally. In the last column is given the ratio of the biological activity of each preparation to its thyroxine content. If it be assumed that L-thyroxine possesses twice the biological activity of DL-thyroxine, this ratio should have a maximum theoretical value of 2.0. However the ratios for the natural thyroid preparations D-1, A, and B were 4.0, 3.1 and 4.5 respectively. The difference between these values and the theoretical is far greater than can be due to experimental error, and indicates that the biological activity of natural thyroid proteins is some four fold greater than the activity of equivalent doses of DL-thyroxine when both are administered intraperitoneally. In Table 3, a ratio of 5.3 was obtained with desiccated thyroid injected subcutaneously in comparison to

[4] We are indebted to Dr. Kenneth W. Thompson of Roche-Organon, Inc., Nutley 10, New Jersey for a generous supply of DL-thyroxine.

DL-thyroxine also subcutaneously administered. The potency of subcutaneously and intraperitoneally administered DL-thyroxine has not been found to differ significantly in these experiments.

These data confirm the work of many earlier investigators who were unable to account for all the thyroxine-like activity of natural

TABLE 2. THE THYROXINE-LIKE ACTIVITY OF NATURAL AND SYNTHETIC THYROACTIVE PROTEINS WHEN ADMINISTERED BY INTRAPERITONEAL INJECTION

0.3% Thiouracil plus	No. of Animals	Administered Dose (Mg.) (100 gm.)	Administered Dose (γ Thyrox.) (100 gm.)	Av. Wt. Animals (gm.)	Average Thyroid Weight (Mg./100 gm.)	Biological Response[3] (γ Thyrox.)	Biological Response Adm. dose
Experiment 1							
Controls	8	—	—	166	36.8±7.9[1]	—	—
DL-thyroxine	6	—	5.00	171	14.2±3.1	5.00	1.00
Desiccated Thyroid-1	5	0.509	1.56[2]	159	9.5±1.8	6.00	3.90
Prod. "B"	5	1.00	1.31	162	9.7±2.2	5.90	4.50
Prod. "A"	5	0.374	1.72	161	12.4±2.8	5.75	3.30
STP-1	6	0.060	1.56	165	28.1±4.8	1.70	1.10
L-Diiodotyrosine	4	25.0	—	150	10.8±1.3	5.70	—
Experiment 2							
Controls	6	—	—	147	33.0±2.6	—	—
DL-thyroxine	6	—	6.00	176	9.3±1.8	6.00	1.00
DesiccatedThyroid-1	6	0.539	1.40	192	10.4±1.9	5.60	4.10
Prod. "B"	6	0.840	1.10	184	14.8±1.3	5.00	4.50
Prod. "A"	6	0.391	1.80	192	10.5±1.4	6.20	3.20
STP-1	6	0.193	5.00	193	11.6±1.7	5.60	1.10
L-Diiodotyrosine	6	15.0	—	153	17.8±3.2	4.20	—
Experiment 3							
Controls	8	—	—	192	27.5±4.3	—	—
DL-thyroxine	5	—	4.00	182	10.6±1.8	4.00	1.00
Desiccated Thyroid-1	5	0.431	1.12	168	8.6±0.4	4.40	3.90
Desiccated Thyroid-2	5	0.362	1.12	182	10.3±1.1	4.00	3.60
Prod."B"	5	0.763	1.00	169	8.7±2.5	4.35	4.40
Prod. "A"	6	0.307	1.41	178	10.3±2.9	4.10	2.90
STP-1	5	0.164	4.10	167	8.9±1.7	4.30	1.00
STP-2	7	0.146	3.50	169	14.0±4.2	3.15	0.90
STP-3	4	0.121	3.50	188	14.5±3.0	3.00	0.85
STP-4	6	0.152	3.50	166	13.2±3.4	3.35	0.95
IC-1	5	2.60	—	171	29.0±3.4	0.00	0.00
Normal Controls							
No Thiouracil	9	—	—	230	9.5±1.3	—	—

[1] Average deviation.
[2] Based on chemical analysis.
[3] Estimated to the nearest 0.05γ from DL-thyroxine dose (I.P.)-response curve for particular experiment.

thyroid proteins in terms of their thyroxine content. Foster and coworkers (1936), however, have reported that the activity of natural thyroid proteins is accounted for if it be assumed that D-thyroxine is inactive and that all the thyroxine present in the thyroid proteins is in the L-form. However, these findings were based on a comparison of orally administered thyroid protein with injected thyroxine. Preliminary data obtained by us indicate that orally administered natural thyroid preparations are appreciably less potent than when they are given parenterally, making it clear that comparison of activities should only be made when both materials are given by the same route. When this is done, a striking excess activity is observed for natural thyroid proteins.

Thyroxine-like activity of parenterally administered synthetic thyroid proteins. In contrast to the results obtained for natural thyroid proteins, synthetic thyroproteins showed less thyroxine-like activity than expected from their L-thyroxine content, the ratio of biological activity to chemical thyroxine content averaging 1.0. It is not believed that the preparation of iodinated proteins involves any extensive racemization of the L-thyroxine formed. Indeed, Reineke and Turner (1943) have isolated L-thyroxine from similarly prepared materials. Thus, the data presented in Tables 2 and 3 leads to the conclusion that the thyroxine-like activity of parenterally administered synthetic thyroproteins is less than that predicted from their L-thyroxine content. This finding conflicts somewhat with the extensive data of Reineke et al. (1945) who reported that in a group of 15 thyroactive iodinated casein preparations the chemical thyroxine content was only about 10 % greater than the biological assay of L-thyroxine as determined from their influence on the metabolic rates of guinea pigs. This would correspond to a ratio of 1.8 as defined above. The possibility that our chemically determined thyroxine values are higher than those actually existing in the iodinated casein has not been sufficiently excluded, however, and this may account for our ratios of less than 2.0. Preliminary tests have indicated a greatly diminished oral activity of thyroactive iodinated casein in the rat, the thyroxine-like activity being approximately 15% of the same dose administered intraperitoneally. This is in qualitative agreement with the observations of Turner and Reineke (1946) on the utilization of synthetic thyroproteins by sheep.

TABLE 3. THE THYROXINE-LIKE ACTIVITY OF NATURAL AND SYNTHETIC THYROID PROTEINS WHEN ADMINISTERED BY SUBCUTANEOUS INJECTION

0.3% Thiouracil plus	No. of Animals	Administered Dose (Mg.) (100 gm.)	Administered Dose (γ Thyrox.) (100 gm.)	Av. Wt. Animals (gm.)	Average Thyroid Weight (Mg./100 gm.)	Biological Response[3] (γ Thyrox.)	Biological Response Adm. dose
Thiouracil Controls	6	—	—	130	30.0±2.9[1]	—	—
DL-thyroxine	6	—	2.00	132	24.4±2.4[1]	2.00	1.0
DL-thyroxine	6	—	4.00	127	13.0±2.2	4.00	1.0
STP-1	6	0.135	3.50[2]	134	21.3±2.4	2.20	0.65
Desiccated Thyroid-1	4	0.336	0.875[2]	133	11.0±0.3	4.60	5.30

[1] Average deviation.
[2] Based on chemical analysis for thyroxine.
[3] Estimated to the nearest 0.05γ from DL-thyroxine dose (subcutaneous)-response curve for particular experiment.

DISCUSSION

There are several possible explanations which might account for the excess parenteral potency of natural thyroproteins as compared to crystalline thyroxine.

Differences in rates of absorption, excretion, and destruction of

thyroxine in natural and synthetic thyroid proteins and in the DL-thyroxine standard may be the explanation of these observations. It is not unlikely that the rate of utilization of proteins and free amino acids when given by this route may vary considerably, and differences in excretory rates rates are equally possible. The order of solubility of thyroactive proteins at pH 8.0 ± 0.5 was STP > A > D $\geqq$ B; while the order of activity was B > D > A > STP. This might be interpreted as relatively slower, more effective parenteral release of the less soluble natural thyroactive poteins.

The possibility of a rather specific synergism between thyroxine and the diiodotyrosine-like fractions of natural thyroid proteins has been postulated by many investigators. Studies of thyroxine-like activity of 3,5-diiodo-L-tyrosine indicate that while this substance is active in the goiter prevention test, it is less than 0.1% as active as DL-thyroxine as shown in table 2. Thus, the greater activity of natural thyroid proteins cannot be accounted for directly by their diiodotyrosine content. There was no evidence of any thyroxine-like activity of diiodotyrosine in IC-1 when given at a level of 0.21 mg. diiodotyrosine equivalent/100 grams of body weight. The diiodotyrosine-thyroxine ratio was as great as 5:1 in the STP preparations with no indication of extra-thyroxine potency. Thus, only a very special synergism, perhaps dependent upon ease of *in vivo* conversion of diiodotyrosine to thyroxine or a thyroxine-diiodotyrosine peptide, could explain the observed greater activity of natural thyroid proteins on the basis of the non-thyroxine iodine fractions.

Harington and Randall (1929) have been able to account for the organic iodine of the thyroid in the forms of 3,5-diiodo-L-tyrosine and L-thyroxine, suggesting that other iodine containing compounds with thyroxine-like activity do not exist in the thyroid gland. However, no decisive information can yet be marshalled to exclude or accept the suggestion that peptides containing both thyroxine and diiodotyrosine may be more active than thyroxine alone. An argument against this possibility is the recent observation of Taurog and Chaikoff (1948) in which free thyroxine has been demonstrated in rat plasma.

The possibility also exists that hormones unrelated to thyroxine which affect metabolism or the pituitary-thyroid axis may occur in the thyroid gland (Mansfeld, 1943), and thus result in the excess potency observed in this work.

SUMMARY

The goiter prevention method has been employed to determine the thyroxine-like activity of parenterally administered natural and synthetic thyroid proteins. Natural thyroid proteins have consistently shown more thyroxine-like activity than could result from their thyroxine content, even assuming that L-thyroxine possesses twice

the activity of DL-thyroxine. However, when compared on the same basis, synthetic thyroproteins have revealed only one-half the parenteral activity expected from their L-thyroxine content as determined by the available chemical methods.

Suggested explanations of these effects include possible differences in rates of absorption, utilization and excretion of the thyroactive proteins and the DL-thyroxine comparison standard, a possible specific synergism of diiodotyrosine-like and thyroxine-like fractions of natural thyroid proteins, and the possible presence of thyroid hormones unrelated to thyroxine which affect the pituitary-thyroid axis.

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A MODIFIED TURBIDIMETRIC METHOD FOR THE ASSAY OF HYALURONIDASE

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WITH the possible exception of the turbidimetric assay, no reliable or practical method exists for the accurate determination of the potency of hyaluronidase preparations. Although the turbidimetric method is by no means ideal, it does lend itself to the performance of a large series of tests and it is accurate to within 10 per cent (Meyer, 1947).

A modification of the turbidimetric hyaluronidase assay is being used in this laboratory with good results. It is based on the turbidimetric method as developed by Seastone (1939, 1943), Kass and Seastone (1944), and Leonard, Perlman and Kurzrok (1946). The modification in assay procedure is the result of the daily handling for approximately eleven months of 10 to 20 assays under practical working conditions. With careful attention to details, the method is accurate to within ± 5 per cent.

MATERIALS AND METHODS

Standard hyaluronidase preparations. In order to minimize day-to-day deviation of assay values, a dry hyaluronidase preparation (bull testis) is set aside as the standard enzyme. The preparation is run side by side with the unknown test solutions. For convenience, the standard is assigned an arbitary potency of *1.0 turbidity reducing unit per milligram.* A standard curve is obtained by plotting the average of two turbidity readings against five enzyme concentrations (.05, .04, .03, .02, .01 mg/ml). A second standard, the potency of which was established by repeated tests, is also assayed daily with the unknowns. It is, therefore, possible to determine the accuracy of the results on any one day. The potencies of the enzyme samples are computed by comparison with the standard curve. The turbidity readings are then converted to milligrams as compared with the standard (see table 1).

Estimation of hyaluronidase activity. For the determination of enzyme activity, a dry hyaluronidase sample, previously diluted to 1 mg/ml with distilled water, is maintained at 24°C. for 30 minutes. The hyaluronidase is further diluted with 0.02 M acetate buffer pH 6.0 to the appropriate dilution range. The varying concentrations of enzyme, in duplicate, are now distributed in a series of tubes and the volume is adjusted to 0.5 ml with buffer solution. To each tube is added 0.5 ml of a purified potassium hyaluronate (umbilical cord) dissolved in 0.1 M acetate buffer pH 6.0 to a concentration

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TABLE 1. ILLUSTRATING METHOD USED FOR THE CALCULATION OF HYALURONIDASE ACTIVITY (See Fig. 1)

Enzyme concentrations (mg./ml.)	.05	.04	.03	.02	.01
Turbidity readings (unknown)	2.5	10	21	32.5	46.5
Standard curve conversion (mg.)	—	.05	.0384	.0264	.0134
Corrected enzyme conc. per milligram	—	1.25	1.28	1.32	1.33

Results: 129.5 TRU/mg.

of 0.2 mg/ml. The tubes are thoroughly mixed, incubated in a 37°C. water bath for 30 minutes and immersed in an ice bath for 5 minutes. To each tube 3 cc of 0.5 M acetate buffer pH 4.2 and 1 cc of 1:5 dilution of horse serum pH 4.2 are added and mixed. The turbidities are allowed to develop for 30° minutes in a 24°C. incubator and read immediately in a Klett-Summerson Photoelectric Colorimeter with a red filter No. 66.

RESULTS

Stability of hyaluronate-hyaluronidase-serum mixture. The instability of the enzyme-substrate-serum precipitate during the time

TABLE 2. STABILITY STUDY OF HYALURONATE-HYALURONIDASE SERUM MIXTURE

Hyaluronidase sample	Turbidity reducing units per milligram					Per cent change in activity			
	Exposure time (minutes)								
	0	10	60	90	150	10	60	90	150
5 C									
A	1.00	1.17	1.34	1.54	1.62	17	34	54	62
B	2.04	2.31	2.75	2.91	2.97	12	34	42	45
C	6.89	6.77	8.18	8.30	9.37	−2	18	20	36
24 C.									
A	1.00	1.05	1.25	1.49	1.54	5	25	49	54
B	1.96	2.00	2.44	2.49	2.90	2	24	27	48
C	6.51	6.98	8.34	8.87	9.06	6	28	36	39

which elapses between sampling and reading the turbidity was reported by Kass and Seastone (1944). These authors found a 15 per cent decrease in turbidity after 2 hours standing at a room temperature. The observations have been confirmed in our laboratory. It was found that the changes occur most frequently in systems containing highly active hyaluronidase preparations. Table 2 summarizes the stability of three hyaluronidase preparations of different purity which were incubated with the standard amount of potassium hyaluronate-acidified serum for the usual 30 minute period (24°C.) directly incubated at 24°C. and 5°C. and assayed at intervals over a period of 2½ hours. The results indicate a marked decrease in turbidity, i.e., increase in activity, of partially purified hyaluronidase A that ranges from 5 to 62 per cent. The more purified preparations B and C show changes between 2 and 48 per cent. It is noted that the intensity of turbidity change decreases at 5°C. with purity of enzyme.

Since the time consumed in reading the turbidities of 15 duplicate samples is approximately 75 minutes, the performance of a large number of tests on any one day was jeopardized by the misleading assay values. This discrepancy was eliminated by the adoption of a "stagger" plan, i.e., two samples in duplicate were diluted, mixed with the standard amount of polysaccharide and incubated at 37°C.

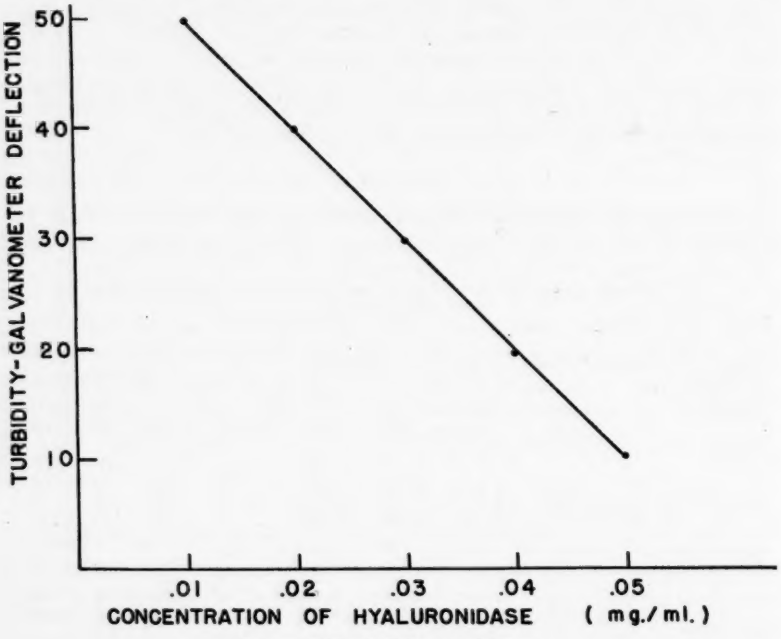

FIG. 1. A Typical Standard Curve

in a water bath; a 10 minute interval was permitted to elapse before the remaining samples, in groups of two, were similarly set up. This modification standardizes the time factor between sampling and reading the turbidity in a large series of assays. The results on any given preparation were found to be highly reproducible.

DISCUSSION

The turbidimetric method for the assay of hyaluronidase defines a *turbidity reducing unit* (TRU) as that amount of enzyme which reduces the turbidity given by 0.2 mg of hyaluronic acid to that given by 0.1 mg (Kass and Seastone, 1944; Leonard Perlman and Kurzrok, 1946; Meyer, 1947). However, samples of purified hyaluronic acid fractionated in this laboratory and in other laboratories were found to possess variable reaction rates with the same concen-

trations of standard enzyme under similar experimental conditions. It is therefore obvious that a hyaluronidase or hyaluronic acid standard is essential to maintain a TRU of comparable value.

In the daily performance of a large series of assays, an adequate supply of hyaluronic acid for an extended period of time was not available. This necessitated the utilization of several batches of the substrate with varied depolymerization properties. In order to minimize these uncontrollable fluctations and at the same time establish a unit of comparative value, one partially purified hyaluronidase preparation was assigned an arbitrary potency of 1.0 TRU/mg and retained as the standard. The enzyme activity of the unknowns may then be expressed in TRU/mg equivalent of standard irrespective of the quality of the substrate. A second standard not only served as a check on the reacting substances in the test but also indicated the accuracy of the method by establishing on the basis of 150 assays an experimental error of ± 5 per cent.

SUMMARY

A turbidimetric assay for determining the hyaluronidase activity of tissue extracts is described. The method simplifies involved procedures, is accurate to within 5 per cent and appears to be of value in the performance of a large number of assays. The activity of unknowns is determined by comparison with a standard hyaluronidase preparation.

The adoption of a "stagger" plan eliminated discrepancies in assay values which resulted whenever a substantial time interval elapsed between sampling and reading turbidities.

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THE EFFECTS OF PITUITARY AND NON-PITUITARY GLAND FACTORS ON THE FORMATION OF INTRACELLULAR COLLOID DROPLETS IN THE THYROID EPITHELIUM OF HYPOPHYSECTOMIZED RATS[1]

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THE PRESENCE of droplets of colloid within the thyroid epithelium was first described in 1872 by Verson in fresh tissue. They were soon after described in fixed thyroid tissue by Gutknecht (1885) and Biondi (1888, 1892). Early workers described their occurrence following the injection of various chemicals (Andersson, 1894; Hurthle, 1894; Galeoti, 1897), or after certain operative procedures unrelated to the thyroid or pituitary glands (Hurthle, 1894; Thomas, 1934). More recent work established a relationship between the formation of intracellular colloid droplets and thyrotrophic hormone. Droplets form promptly after the administration of exogenous thyrotrophic hormone to intact animals (Grant, 1930, 1931; Severinghaus, 1933; Uhlenhuth, 1936; Okkels, 1934; Ponse, 1938; Aleschin, 1935, 1939; Ponse and Altschuler, 1940; De Robertis, 1941, 1942) with dosages of hormone smaller than those needed to increase thyroid cell height of gland weight (De Robertis and Del Conte, 1944; Dvoskin, 1947b). They appear also after presumably enhanced endogenous thyrotrophic hormone production caused by partial thyroidectomy (Hurthle, 1894; Tschassovnikoff, 1915; Ishimaru, 1926; Satwornitzkaja and Simnitsky, 1932) and by thiourea or thiouracil administration (Thomas, 1944; Grasso, 1946; Dvoskin, 1947c.)

However, droplet formation may not always be a specific response to thyrotrophic hormone. Thus Langendorff (1889), Lubcke (1902), Champy (1915), Demuth (1932) and, more recently, Dvoskin (1947d) demonstrated that, in vitro, colloid infiltrates the epithelium and forms intracellular colloid droplets in the absence of added thyrotrophic hormone. This does not deny the fact that droplet formation may also be induced in vitro under certain conditions with thyrotrophic hormone (Junqueira, 1947; Dvoskin, 1947d), but suggests that this response lacks specificity for thyrotrophic hormone.

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The following experiments were undertaken in order to study the factors related to droplet formation in the thyroid of living animals.

METHODS

The first group of experiments was performed on hypophysectomized rats in order to rule out the possibility of participation of the test animals' own pituitary gland in the reaction. Male rats of the Long-Evans strain, ranging from 50 to 80 days of age, were hypophysectomized by the parapharyngeal approach. Completeness of removal of the hypophysis was ascertained in all cases by study of serial sections of the pituitary capsules. The solutions tested were administered by intracardiac injection one day after operation. One series of experiments was designed to determine the influence of time after injection on the response due to a fixed dosage of pituitary extract. Since the maximum number of droplets formed one hour after injection, another series of experiments was performed to study the response due to various dosages of this pituitary extract[3] and of a standard pituitary gland preparation[4] at one hour after injection. Since thyroxine was later to be used to inhibit the secretion of thyrotrophic hormone in intact animals, it was important to determine whether it or iodide could directly inhibit droplet formation in the thyroid of hypophysectomized rats due to simultaneously administered thyrotrophic hormone.

The specificity of droplet formation by thyrotrophic hormone was tested by administering toxic dosages of typhoid vaccine or histamine diphosphate or pilocarpine hydrochloride to hypophysectomized rats, and studying the effect on droplet formation one hour after the first injection. Typhoid vaccine and the lower dosages of histamine and pilocarpine were administered by a single intra-cardiac injection. The higher dosages of histamine and pilocarpine were administered in divided dosages over an hour period. Half the dosage was administered by intracardiac injection. The remainder was given subsequently by intraperitoneal injection.

Another series of experiments was performed to determine the number of droplets which formed at 1, 8 or 20 days after hypophysectomy. These animals were given an intracardiac injection of 50 μg. of hog pituitary extract or 1 cc. of typhoid vaccine and autopsied one hour later.

Fresh unmodified pooled sera from rats subjected to the following experimental conditions were injected into the heart of hypophysectomized rats in 2 cc. amounts in order to determine the effect on droplet formation: a. Sera from normal young female rats housed at room temperature. b. Sera from young female rats exposed to 5° C. for 16 hours prior to exsanguination. Exposure to cold (Cramer, 1928; Kenyon, 1933; Kuschinsky, 1935; Woitkewitch, 1935; Baillif, 1937; Uotila, 1939; Starr and Roskelley, 1940) is known to result in stimulation of endogenous thyrotrophic hormone secretion as evidenced by increase in thyroid cell height. c. Some of this serum (b) was

[3] The pituitary extract used was a five-year-old flavianic acid precipitate prepared from hog whole pituitary glands by Dr. George Smelser. This extract contained large amounts of thyrotrophic hormone, and was the same as that assayed on chicks in an earlier paper (Dvoskin, 1947b). A generous supply of this extract was available through the kindness of Dr. Smelser.

[4] Held for the Health Organization of the League of Nations by the National Institute for Medical Research, London, and obtained from the Committee of Revision of the Pharmacopoeia of the United States of America, Philadelphia, Pa.

also incubated at room temperature with thiouracil,[5] in amounts of 1 mg. per cc. of serum, for 30 minutes prior to injection. This was done to determine whether thiouracil could reactivate (Rawson, Albert, McArthur, Merrill, Lennon and Riddell, 1946) or augment (Albert, Rawson, Merrill, Lennon and Riddell, 1947) any thyrotrophic hormone which might be present in the serum. d. Serum from similarly cold exposed rats which had been injected twice subcutaneously with 100 μg. of sodium iodide in 0.2 cc. of water, once at the start and once halfway through the period of cold exposure. This experiment was performed since Rawson, Moore, Peacock, Means, Cope and Riddell (1945) showed that iodide prevented the inactivation of thyrotrophic hormone by thyroid tissue in vitro. e. Sera from thyroidectomized young female rats kept at room temperature for 17 hours or 10 days following thyroidectomy and prior to exsanguination. f. Sera from rats exposed to 5° C. for 17 hours following thyroidectomy, and prior to exsanguination. g. Sera from normal young male rats fed 0.1% thiouracil in the diet for 7 days prior to exsanguination. The feeding of goiterogenic drugs (MacKenzie and MacKenzie, 1943; Astwood, Sullivan, Bissell and Tyslowitz, 1943) is known to stimulate secretion of thyrotrophic hormone as evidenced by increase in thyroid cell height and gland weight.

Human sera, obtained from normal patients and from patients suffering from hyperthyroidism were also injected. The sera from the latter were taken before or after therapy with iodine or thiouracil and also from post-thyroidectomy cases. Since the post-operative human sera were always toxic or lethal to rats, control sera from patients following other operations were tested to determine whether they also stimulated droplet formation. Some of the hypophysectomized rats were injected with 1.0 cc. of Tyrode's solution, and served as injected controls.

In the experiments with normal rats, droplet formation was induced by exposure to cold and by thiouracil feeding. The role of the pituitary gland in the maintenance of the droplets formed by these procedures was studied by hypophysectomizing these animals and then continuing cold-exposure or thiouracil-feeding.

Other experiments were designed to determine whether droplet formation could be induced in the thyroid of the hypophysectomized rat by cold-exposure or thiouracil injections. The increase in thyroid cell height or gland weight does not occur in hypophysectomized animals exposed to cold (Wolf and Greep, 1937; Uotila, 1939) or fed goiterogenic drugs (Astwood, et al., 1943).

The role of the pituitary gland was tested indirectly by studying the effects of thyroxine administration on droplet formation due to concurrent exposure to cold or thiouracil administration in normal rats. Thyroxine administration prevents the increase in thyroid cell height and gland weight in normal rats fed goiterogenic drugs (MacKenzie and MacKenzie, 1943; Astwood, et al., 1943; Reineke, Mixner and Turner, 1945), presumably by suppressing thyrotrophic hormone secretion. Thyroxine was administered in two forms. One group was treated with subcutaneous or intrasplenic implants of 1.4 mg. to 1.8 mg. pellets of mono-sodium d,l-thyroxine.[6] Lower

[5] The thiouracil was supplied through the courtesy of the Calco Chemical Division of the American Cyanamid Company.

[6] The mono-sodium thyroxine was supplied through the courtesy of Hoffmann-LaRoche.

dosages were administered by implanting similar pellets made using cholesterol as a diluent. Since the weight changes in these pellets were very small, the dosages which they provided could only be roughly estimated. In order to study the effect of a known dosage another group received daily subcutaneous injections of 7.5 μg. d,l-thyroxine in aqueous solution. Animals injected daily with 8.9 mg. NaI served as controls for the thyroxine treated animals.

Since the adrenal cortex (Long, 1947; Sayers and Sayers, 1947) and medulla (Cannon, Querido, Britton, and Bright, 1927; Cramer, 1928) are known to be stimulated to secrete by exposure to cold, it was of interest to determine whether droplet formation in the thyroid was related to the activation of this gland. In these experiments, the response of adrenalectomized rats to cold-exposure was studied. In others, the effect of injections of either epinephrine or adrenal cortical extract on droplet formation in normal rats kept at room temperature was determined.

At the end of each experiment, the animals were killed by illuminating gas, the thyroids were immediately dissected free under a binocular microscope, and weighed to the nearest milligram. The glands were then promptly fixed in Carnoy's fluid,[7] imbedded in paraffin, and sectioned at 4 μ. Sections were stained by the modified Heidenhain-Azan stain.

The number of intracellular colloid droplets in the thyroid epithelium of each animal was determined by totalling the number of droplets in the epithelium of 25 cross sections of follicles in a section through the mid-portion of the gland. A 10 × ocular and a 90 × oil immersion objective lens were used. In order to determine if there was any response in cell heights under conditions which were sufficient to stimulate droplet formation, cell heights were measured by a method described earlier (Dvoskin, 1947a, 1947b, 1947c). The mean and standard deviation of the number of colloid droplets and of the cell heights and weights of the thyroids of each group, and P values comparing the means of different groups, were calculated (Fisher, 1944).

RESULTS

1. Effect of Pituitary Extracts in Hypophysectomized Rats. The thyroid epithelium of adult male hypophysectomized rats contained no colloid droplets one day after operation (Fig. 1). A single intracardiac injection of 50 μg. of hog pituitary extract produced large numbers of droplets as early as 30 minutes after injection. The number of droplets was maximal at 60 minutes after injection and only a few droplets could be found after 180 minutes (Table 1).

An injection of 1 μg. of hog pituitary extract did not stimulate droplet formation within one hour after its injection. However, as little as 5 μg. of the extract stimulated the formation of a small number of intracellular colloid droplets (Table 2). Increasing dosages, up to 100 μg., stimulated formation of increasing numbers of droplets (Fig. 2). Statistically significant differences were found between the means of the groups with but slight overlap between groups (Table 2). The difference in cell height between animals treated with

[7] This fixative preserves the intracellular colloid droplets in rat thyroid epithelium.

100 μg. of the hog pituitary extract and that of untreated controls was at the borderline of statistical significance, while the cell height of animals treated with 50 μg. was not significantly increased over the controls.

The International Preparation of the Anterior Pituitary Gland Substance of the Ox was tested for its effect on droplet formation at two dosage levels. The slope of the line which may be plotted from the number of droplets which appeared following injection of this preparation closely resembles that obtained with the hog pituitary

TABLE 1. INTRACELLULAR COLLOID DROPLETS IN THYROID EPITHELIUM OF MALE RATS ONE DAY AFTER HYPOPHYSECTOMY AND AFTER VARYING TIMES AFTER INTRACARDIAC INJECTION WITH 50 μg. OF HOG PITUITARY EXTRACT IN 0.5 CC. OF WATER

Time—min. after injection	No. of animals	No. of droplets in 25 follicles Average $\pm\sigma$	Actual results	P[1]
30	5	387 ± 67	314, 319, 410, 436, 458	
60	5	432 ± 65	352, 393, 397, 509, 509	>0.2
120	5	114 ± 26	0, 10, 12, 26, 66	<0.01
180	5	15 ± 4	0, 0, 2, 3, 10	<0.01

[1] P value of group compared with one treated for 30 minutes.

extract. Comparison of the activity of a solution prepared from 150 μg. of the International Preparation with the dosage-response curve of the hog pituitary preparation shows that it was roughly as active as 3.5 μg. of the hog pituitary extract. The solution prepared from 750 μg. of the International Preparation was roughly as active as 18 μg. of hog pituitary extract. Albert, Rawson, Merrill, Lennon and Riddell (1946) reported that 15 mg. (15,000 μg.) of the International Preparation was equal to one Junkmann-Schoeller Unit. On this basis one may say that this test method is able to detect the activity in a solution prepared from 150 μg. (Table 2) or 0.01 Junkmann-Schoeller Unit.

The administration of 10 μg. of d,1-thyroxine, a dosage which will inhibit droplet formation in normal animals, did not influence droplet formation due to the simulatneous injection of 50 μg. of pituitary extract (Table 2). The injection of 100 μg. of NaI also did not influence the results of injection of pituitary extract (Table 2).

2. Specificity of the Reaction. The injection of 1 cc. of typhoid vaccine invariably resulted in the formation of colloid droplets in the thyroids of hypophysectomized rats (Table 2, Fig. 4). The injection of 1 mg. of histamine stimulated droplet formation in half of the animals treated with this dosage, while 3 mg. of histamine stimulated droplet formation in all animals (Table 2, Fig. 3). Similarly, 1 mg. of pilocarpine elicited droplet formation in one of six animals, while 15 mg. resulted in droplet formation in all animals (Table 2).

TABLE 2. INTRACELLULAR COLLOID DROPLETS OF THYROID EPITHELIUM OF ADULT MALE RATS, ONE DAY AFTER HYPOPHYSECTOMY AND ONE HOUR AFTER THE INTRACARDIAC INJECTION OF THE FOLLOWING MATERIALS

Group	No. of animals	No. of intracellular colloid droplets in 25 follicles Average $\pm\sigma$	Actual results	P[1]
Uninjected Controls	10	0 ± 0	0, 0, 0, 0, 0, 0, 0, 0, 0, 0.	
1 cc. Tyrode's Solution	3	0 ± 0	0, 0, 0.	
1 μg. Hog. Pit. Extr.	5	0 ± 0	0, 0, 0, 0, 0.	
5 μg. Hog. Pit. Extr.	5	25 ± 17	7, 8, 28, 41, 42.	<0.01
10 μg. Hog. Pit. Extr.	5	51 ± 12	38, 40, 48, 63, 65.	<0.01
25 μg. Hog. Pit. Extr.	5	291 ± 69	235, 242, 246, 360, 373.	<0.01
50 μg. Hog. Pit. Extr.	5	432 ± 65	352, 393, 397, 509, 509.	<0.02
100 μg. Hog. Pit. Extr.	5	647 ± 125	510, 526, 670, 692, 839.	<0.02
50 μg. Hog. Pit. Extr. and 10 μg. thyroxine	5	414 ± 66	326, 378, 424, 442, 500.	>0.07
50 μg. Hog. Pit. Extr. and 100 μg. NaI	5	414 ± 37	371, 385, 420, 430, 465.	>0.3
150 μg. Ox. Pit. (Int. Standard)	5	13 ± 6	6, 9, 11, 19, 20.	
750 μg. Ox. Pit. (Int. Standard)	5	174 ± 52	112, 143, 181, 183, 251.	
1 cc. Typhoid Vaccine	6	46 ± 24	17, 21, 44, 55, 56, 82.	
1 mg. Histamine Diphosphate	8	41 ± 67	0, 0, 0, 0, 4, 28, 139, 158.	
3 mg. Histamine Diphosphate	6	46 ± 27	15, 25, 35, 47, 71, 84.	
1 mg. Pilocarpine Hydrochloride	6	5 ± 67	0, 0, 0, 0, 0, 30.	
15 mg. Pilocarpine Hydrochloride	6	33 ± 23	7, 18, 24, 26, 53, 67.	
2 mg. Thiouracil	5	0 ± 0	0, 0, 0, 0, 0.	

[1] P value comparing group with one treated with the next lower dosage. Groups treated with thyroxine or NaI and hog pituitary extract are compared with the one treated with the same amount of pituitary extract alone.

There was a rough correlation between the toxic effect of materials administered and the number of droplets which formed. In general, the larger and more toxic dosages were more effective in stimulating droplet formation than the lower less toxic dosages. In most cases, the injection of a material which prostrated the animals stimulated droplet formation. The droplets which formed after injection of these toxins were most numerous in the peripheral follicles, and were

never as numerous as after injection of the large dosages of non-toxic pituitary extract.

3. Effect of Time After Hypophysectomy on Droplet Formation. The number of droplets which formed one hour after the intracardiac injection of 50 μg. of hog pituitary extract was markedly lower at 8 than at 1 day after hypophysectomy. A further reduction in the

TABLE 3. INTRACELLULAR COLLOID DROPLETS AND CELL HEIGHT OF THYROID EPITHELIUM OF HYPOPHYSECTOMIZED MALE RATS AT VARIOUS TIMES AFTER OPERATION AND ONE HOUR AFTER INJECTION OF PITUITARY EXTRACT OR TYPHOID VACCINE

Treatment	Days after hypophysectomy	No. of animals	No. of droplets in 25 follicles		Cell height—microns Av. ±σ
			Av. ±σ	Counts in individual animals	
50 μg. Hog. Pituitary Extract	1	5	432±65	352, 393, 397, 509, 509.	7.8±0.9
	8	3	222±39	196, 204 268.	4.9±0.2
	20	3	70±22	49, 70, 92.	3.9±0.3
1.0 cc. Typhoid Vaccine	1	6	46±24	17, 21, 44, 55, 56, 82.	7.1±0.5
	8	4	8±5	3, 3, 12, 12.	5.0±0.2
	20	4	0±0	0, 0, 0, 0.	3.4±0.3

number of droplets occurred if a 20 day period elapsed before the injection (Table 3).

A progressive decrease in the number of droplets was also observed following the injection of 1 cc. of typhoid vaccine at increasing periods after hypophysectomy. Indeed, at 20 days after operation, no droplet formation after typhoid injection was observed (Table 3). However, it was difficult to be absolutely certain of this since the nucleoli, which stained like colloid droplets, were abundant in the nuclei which filled the flattened epithelial cells.

4. Effect of Sera. Rat sera were never toxic or lethal when administered to hypophysectomized rats by intracardiac injection. Of the rat sera tested, only those from certain of the thyroidectomized or thiouracil-treated normal rats stimulated droplet formation in the

PLATE 1. Thyroids of hypophysectomized male rats one day after operation and one hour after the intracardiac injection of the following materials. × 445.

FIG. 1. Untreated control.
FIG. 2. 100 μg. of hog pituitary extract in 0.5 cc. of water.
FIG. 3. 1 mg. of histamine diphosphate in 1.0 cc. of water.
FIG. 4. 1 cc. of typhoid vaccine.

Note absence of intracellular colloid droplets in thyroid epithelium in Fig. 1, and presence of droplets in Figs. 2, 3 and 4.

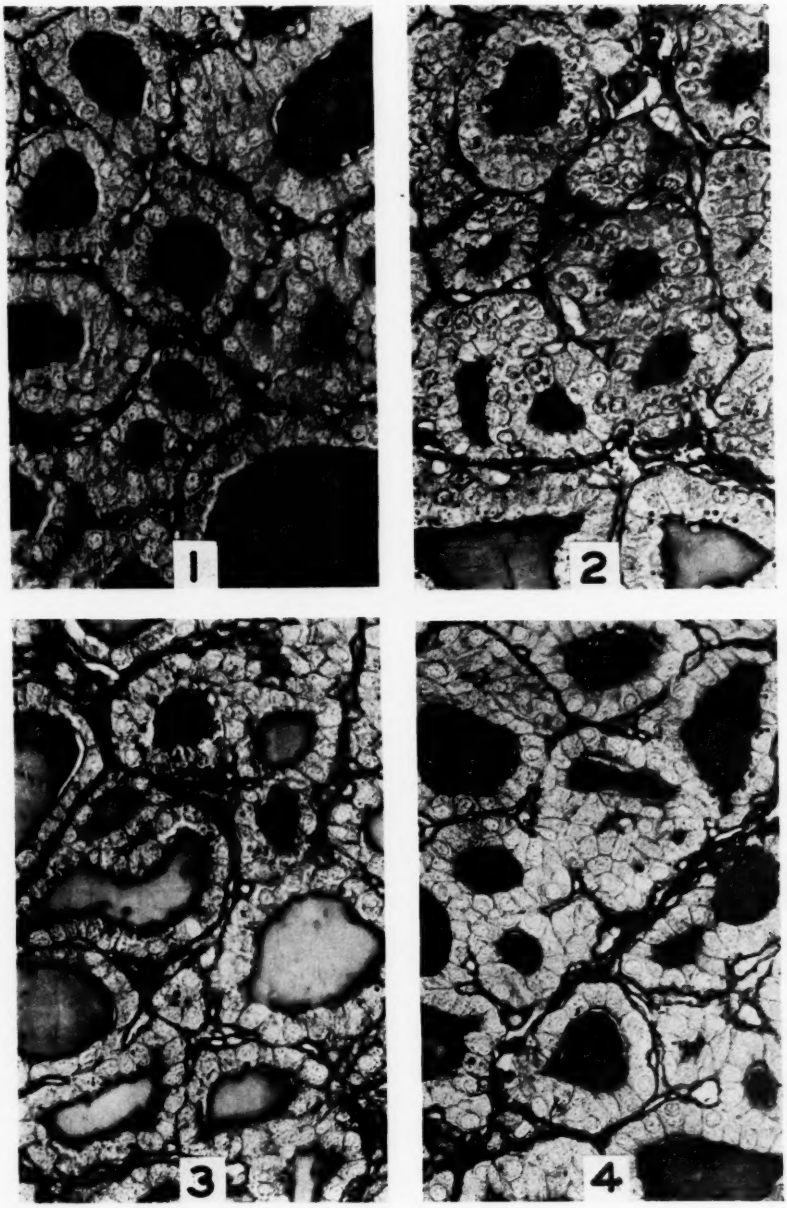

PLATE 1. See opposite page for explanation.

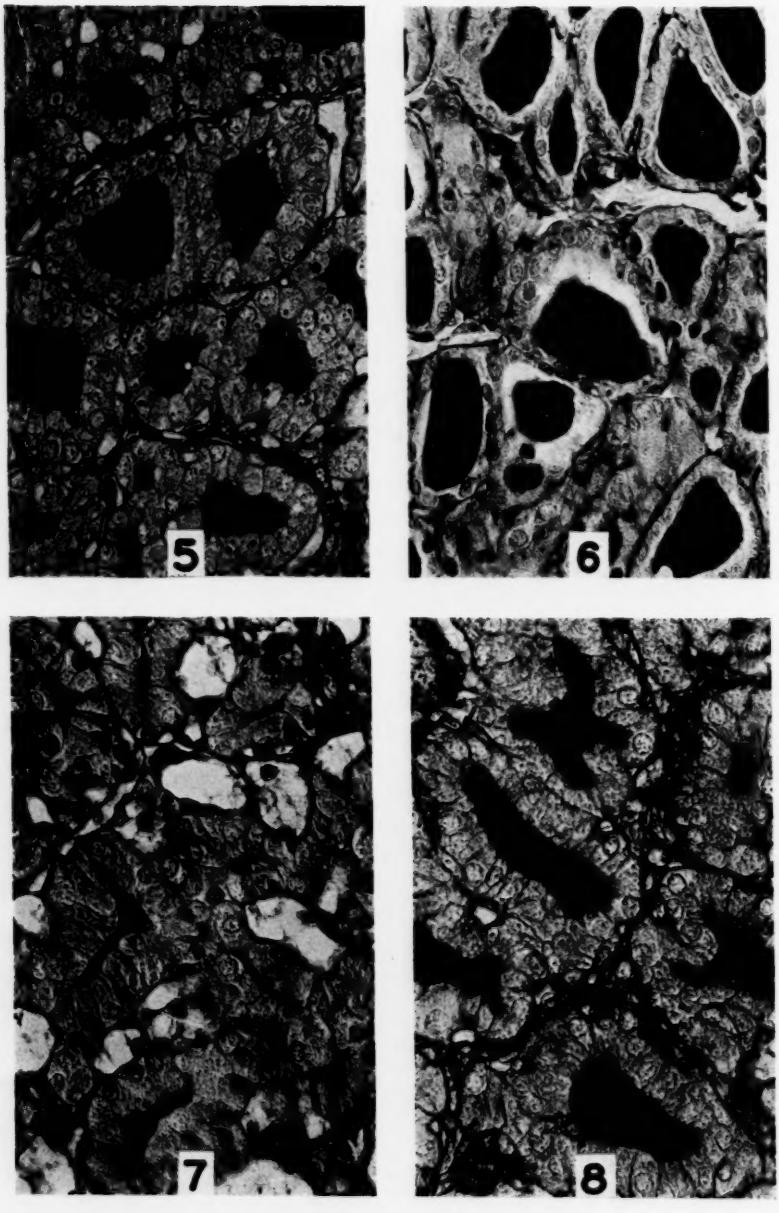

PLATE 2. See opposite page for explanation.

recipient hypophysectomized rats (Table 4). The serum from thyroidectomized rats housed at 5°C. for 17 hours following operation did contain a droplet stimulating material. If the rats were kept at room temperature their sera did not stimulate droplet formation in recipient rats. The serum from rats kept at room temperature for 10

TABLE 4. ASSAY OF SERUM OF THYROIDECTOMIZED OR NORMAL OR THIOURACIL-FED NORMAL YOUNG RATS FOR DROPLET STIMULATING MATERIAL. DONOR RATS WERE KEPT AT VARYING TEMPERATURES FOR VARYING TIMES. ASSAY WAS PERFORMED BY INTRACARDIAC INJECTION OF UNMODIFIED SERUM INTO HYPOPHYSECTOMIZED RATS ONE DAY AFTER OPERATION. ANIMALS WERE AUTOPSIED ONE HOUR AFTER THE INJECTION

Serum assayed					Assayed on		Total no. of colloid droplets in 25 follicles	
Type of animal	Animal kept Temp. °C.	For time	Vol. of pooled serum cc.	From animal #	No. of rats	Vol. Inj. per animal cc.	Donor	Recipient
Normal	25		4	2	2	2.0	13, 59.	0, 0.
Normal	5	16 hrs.	9	5	4	2.0	109, 117, 226, 279, 339.	0, 0, 0, 0.
Normal	5	16 hrs.	6[1]	3	2	2.0	32, 149, 194.	0, 0.
Normal[2]	5	16 hrs.	8	4	3	2.0	12, 17, 63, 98.	0, 0, 0.
Thrx.	25	17 hrs.	5	3	2	2.0		0, 0.
Thyrx.	5	17 hrs.	5	3	2	2.0		143, 158.
Thyrx.	25	10 d.	4	2	2	1.7		28, 36.
Normal Thiouracil-Fed	25	7 d.	8	4	4	2.0	138, 146, 147, 175.	135, 169, 177, 245.

[1] 4 cc. serum mixed with 4 mg. thiouracil, and kept at 25°C. for 30 minutes prior to assay.
[2] Donor animals injected subcutaneously with 200 μg. NaI in 0.2 cc. at start and again midway through period of exposure to cold.

days following thyroidectomy stimulated formation of small numbers of droplets in the thyroids of the recipient animals (Table 4). Although droplet formation was stimulated in the thyroids of normal rats exposed to cold, their sera did not stimulate droplet formation in recipient hypophysectomized rats. Nor did treatment of the donor normal cold-exposed rats with iodide permit such a material to be demonstrated. Nor did the serum of cold-exposed normal rats contain an agent which could be rendered active by incubation with thiouracil prior to injection into hypopysectomized rats. The serum from rats fed thiouracil for 7 days contained a droplet stimulating agent, and droplets were also present in the thyroids of the donor rats (Table 4). This was not due to the presence of thiouracil in such serum, since injection of thiouracil did not induce droplet formation in the thyroids of hypophysectomized rats (Table 2).

PLATE 2. Thyroids of young female rats. × 465.

FIG. 5. Exposed to 5° C. for 2 hours before autopsy.

FIG. 6. Fed. 0.1% thiouracil and treated with a 1.5 mg. pellet of mono-sodium d, l-thyroxine for 10 days. Note the intracellular colloid droplets in the follicles in the upper right hand corner of the figure.

FIG. 7. Fed 0.1% thiouracil for 10 days.

FIG. 8. Fed 0.1% thiouracil for 11 days, and hypophysectomized one day before autopsy.

Most of the human sera, and particularly those of post-operative patients, were toxic and even lethal to the test animals. Positive responses were frequent and seemed unrelated to the clinical status of the donor individual. Droplet formation was occasionally stimulated by injection of serum from normal individuals or from non-thyroid post-operative cases (Table 5). The numbers of droplets which formed seemed related to toxicity of the sera in the normal group. Although droplet formation was usually prominent following the in-

TABLE 5. INTRACELLULAR COLLOID DROPLETS OF THYROID EPITHELIUM OF ADULT RATS, ONE DAY AFTER HYPOPHYSECTOMY AND ONE HOUR AFTER THE INTRACARDIAC INJECTION OF 2 CC. OF SERUM FROM PATIENTS WITH THE FOLLOWING THYROID DISEASES

Diagnosis	Normal	Untreated hyperthyroid	Iodine-treated hyperthyroid	Thiouracil-treated hyperthyroid	Post thyroidectomy	Post operative (not thyroidectomy)
No. of Colloid Droplets in 25 Follicles Actual Results	0, 0. 0, 0. 0, 0, 0. 0, 0, 0. 0, 4. 0, 5. 16, 37, 56. 30, 51. 65, 79.	0, 0, 0, 12. 14, 15, 25, 73. 32, 60, 101.	0, 15, 22. 17, 19, 47. 7, 32, 40, 45, 115.	0, 20. 14, 26.	8, 9. 14, 29, 36. 31, 32, 40. 40, 78. 46, 75, 113, 172. 135. 121, 137, 147.	2, 4. 9. 45, 74.
Average No. of Droplets and Toxicity[1]	0 0 0 0 0 0 0 0 2 0 3 0 36 +++ 41 ++ 72 ++	6 + 32 +++ 64 +	12 + 28 +++ 48 +	10 + 23 +	9 ++++ 28 +++ 34 +++ 59 +++ 101 +++ 135 ++++ 135 +++	3 +++ 9 +++ 60 +++

[1] 0 non-toxic.
+ slightly toxic.
++ somewhat toxic.
+++ toxic and lethal to some rats.
++++ lethal to all rats.

jection of the more toxic sera in the other groups, there were some sera which were very toxic and which stimulated the formation of but few droplets (Table 5).

5. Effect of Hypophysectomy on Droplet Formation. A group of 12 normal rats were exposed to 5°C. for 2 hours. At this time the left lobe of the thyroid of each animal was removed. These lobes contained an average of 70 droplets with a range of from 16 to 175. Hypophysectomy was performed at this time in half of these animals, and the pituitary glands were not removed in the other half. Exposure to cold was continued for an additional 16 hours. At the end of this time, the right lobe of the thyroid of each hypophysectomized animal was completely free of colloid droplets, while the thyroids of the animals with intact pituitary glands contained an average of 66 droplets with a range of from 23 to 147 colloid droplets.

Hypophysectomized rats, exposed to 5°C. for 2 hours one day following operation, did not form any colloid droplets in their thyroids (Table 6).

TABLE 6. INTRACELLULAR COLLOID DROPLETS AND CELL HEIGHT OF THYROID EPITHELIUM OF YOUNG FEMALE RATS EXPOSED TO 5°C. FOR 2 OR 4 OR 16 HOURS. SOME COLD EXPOSED ANIMALS WERE PRETREATED WITH A SINGLE SUBCUTANEOUS INJECTION OF 100 μg. NaI OR 10 μg. d,l-THYROXINE. ONE GROUP WAS HYPOPHYSECTOMIZED (HYPOX.) AND ANOTHER GROUP WAS ADRENALECTOMIZED (ADRENX.) ONE DAY PRIOR TO COLD EXPOSURE. ONE GROUP OF NORMAL RATS, KEPT AT ROOM TEMPERATURE (25°C.), WAS INJECTED SUBCUTANEOUSLY FOUR TIMES, EACH AT ½ HR. INTERVALS, WITH 0.05 CC. OF 1:1000 EPINEPHRINE HYDROCHLORIDE, BEFORE AUTOPSY. ANOTHER GROUP WAS INJECTED SUBCUTANEOUSLY TWICE, EACH AT HOURLY INTERVALS, WITH 1.0 CC. OF ADRENAL CORTICAL EXTRACT (A.C. EX.) BEFORE AUTOPSY

Treatment	No. of animal	Total no. of intracellular colld droplets in 25 follicles					Cell height—μ
		Av. ±σ	Ratio control −1.00	P	P_1	Actual results	Av. ±σ
Untreated Controls	13	8.4± 23.2	1.00			0, 0, 0, 0, 0, 0, 0, 1, 2, 3, 3, 16, 84.	7.19±0.54
5°C.—2 hrs. Controls	7	248.2±127.1	29.55		<0.01	58, 105, 140, 221, 306, 349, 368.	8.10±0.36
5°C.—2 hrs. 100 μg. NaI.	11	170.9±148.8	20.35	>0.3		0, 0, 0, 19, 37, 176, 206, 213, 305, 411, 513.	7.62±0.90
5°C.—2 hrs. 10 μg. thyrox.	12	17.9± 42.6	2.13	<0.01	<0.01	0, 0, 0, 0, 1, 1, 2, 2, 3, 3, 60, 143.	7.97±0.57
5°C.—4 hrs. Controls	7	96.0±104.8	11.43		<0.01	0, 12, 50, 116, 133, 141, 220.	7.49±0.75
5°C.—4 hrs. 100 μg. NaI.	6	80.0± 52.1	9.52	>0.7		7, 41, 70, 85, 103, 174.	7.83±0.55
5°C.—4 hrs. 10 μg. thyrox.	7	0.7± 1.5	0.08	<0.05	<0.01	0, 0, 0, 0, 0, 1, 4.	7.00±0.67
5°C.—2 hrs. Hypox.	6	0.0± 0.0	0.00			0, 0, 0, 0, 0, 0.	6.97±0.37
Adrenx. Controls	4	21.5± 29.4	2.56			0, 10, 11, 65.	8.00±0.46
5°C.—2 hrs. Adrenx.	4	194.0± 88.4	24.05	<0.01	<0.01	64, 217, 235, 260.	7.95±0.39
Epinephrine 0.2 mg.	6	1.0± 1.2	0.12			0, 0, 0, 1, 2, 3.	7.73±0.58
A.C. Extract 2.0 cc.	4	14.3± 17.3	1.70			0, 0, 22, 35.	8.50±0.30
5°C.—16 hrs. Controls	8	180.6± 99.1	21.50		<0.01	32, 109, 117, 149, 194, 226, 279, 339.	8.20±0.31

P —P values are those comparing the group with the corresponding control cold exposed group.
P_1—P values are those comparing the group with the corresponding NaI treated group.
The control cold exposed groups are compared with the untreated controls.

A group of normal rats were fed a diet containing 0.1% thiouracil for 10 or 14 days. Their thyroids contained numerous colloid droplets in the tall thyroid epithelium (Table 7; Fig. 7). Hypophysectomy, performed after 10 days of thiouracil feeding in one group of rats, resulted in the complete disappearance of colloid droplets as early as one day after operation, despite continuation of thiouracil feeding (Table 7; Fig. 8). No new droplets formed during a 4 day period following hypophysectomy, despite continuation of thiouracil feeding (Table 7). Thyroid gland cell height and weight declined progressively following operation (Table 7).

6. Effect of Thyroxine and Iodide Administration on Droplet Formation. Exposure of normal rats to 5°C. for 2 or 4 or 16 hours resulted in a marked increase in the number of colloid droplets in the

thyroid epithelium (Table 6; Fig. 5). Pre-treatment of normal rats with 10 μg. of d,l-thyroxine immediately prior to exposure to 5°C. for 2 or 4 hours prevented this increase in 17 of 19 animals (Table 6). Pre-treatment with 100 μg. of NaI did not prevent an increase in the number of colloid droplets due to exposure to 5°C. for 2 or 4 hours in 13 of 17 animals (Table 6). Thyroid cell heights were not significantly altered by these treatments.

Feeding normal rats a diet containing 0.1% thiouracil for 10 days produced a marked increase in the number of intracellular colloid droplets, as well as an increase in thyroid cell height and gland weight.

TABLE 7. WEIGHT, CELL HEIGHT AND INTRACELLULAR COLLOID DROPLETS OF THYROIDS OF YOUNG FEMALE RATS FED 0.1% THIOURACIL IN THE DIET FOR FROM 10 TO 14 DAYS. ONE GROUP OF RATS WAS HYPOPHYSECTOMIZED (HYPX.) AFTER 10 DAYS OF THIOURACIL FEEDING. GROUPS WERE AUTOPSIED 1, 2, 3 AND 4 DAYS FOLLOWING THIS OPERATION. THIOURACIL FEEDING CONTINUED DURING THE POST-OPERATIVE PERIOD. OTHER RATS WERE INJECTED SUBCUTANEOUSLY EACH DAY WITH NAI OR THYROXINE DURING A 14 DAY PERIOD OF THIOURACIL FEEDING

Group	No. of animals	Thyroid weight—mg./100 gm. body wt.		Cell height—microns		No. of colloid droplets in 25 follicles	
		Average	Range	Average	Range	Average	Range
Unoperated thiouracil-fed 10 days	2	19.9	17.1–22.8	10.1	9.8–10.4	194	185–203
1 day after HypX.	2	21.7	21.3–22.2	9.8	9.7– 9.8	0	0
2 days after HypX.	2	16.3	15.9–16.7	8.1	7.9– 8.3	0	0
3 days after HypX.	2	15.0	13.8–16.2	7.9	7.6–8.2	0	0
4 days after HypX.	2	13.0	12.4–13.5	7.6	7.4– 7.7	0	0
7.5 μg. d-l thyroxine/d. thiouracil-fed 14 days	4	5.8	4.5– 7.5	5.4	5.2– 6.1	22	14–34
8.9 μg. NaI/d. thiouracil-fed 14 days	7	18.7	16.8–20.7	13.7	12.9–14.2	244	155–371
Untreated normal controls	5	7.7	7.0– 9.2	8.0	7.8– 8.6	16	12–33
Unoperated thiouracil-fed 14 days	4	22.9	19.3–25.6	13.5	13.2–13.9	231	163–310

The number of intracellular colloid droplets and the cell heights and weights of the thyroids were lowered with dosages of 20 μg. or 50 μg. of thyroxine per day from pellets containing mono-sodium d,l-thyroxine. The latter dosage reduced thyroid cell height and gland weight to levels even lower than those of untreated controls. However, even this large dosage did not completely prevent the appearance of intracellular colloid droplets in the thyroid epithelium of thiouracil-fed rats (Table 8; Fig. 6).

The daily subcutaneous injection of a solution containing 7.5 μg. of d,l-thyroxine in rats fed 0.1% thiouracil in the diet for 14 days prevented the marked increase in the number of colloid droplets which resulted from feeding thiouracil alone (Table 7). However, droplet formation was not completely inhibited, and small numbers of droplets were present in the thyroids of thyroxine-injected rats (Table 7).

TABLE 8. WEIGHT, CELL HEIGHT AND INTRACELLULAR COLLOID DROPLETS OF THYROIDS OF YOUNG FEMALE RATS FED 0.1% THIOURACIL FOR 10 DAYS AND TREATED WITH PELLETS COMPOSED OF MONO-SODIUM d,l-THYROXINE (M.S.Th.) OR MONO-SODIUM d,l-THYROXINE AND CHOLESTEROL (CHOL)

Treatment		No. of animals	Thyroid weight mg./100 gm. Body wt.			Cell height—microns			No. of colloid droplets in 25 follicles		
Type of pellet	Thyroxine[1] μg./d.		Av. ±σ	Ratio control	P[2]	Av. ±σ	Ratio control	P[2]	Av. ±σ	Ratio control	P[2]
100% M.S.Th.	50	4	7.4±0.8	0.63	<0.01	4.9±0.0	0.57	<0.01	20± 12	200	<0.01
25% M.S.Th. 75% Chol.	20	4	10.8±4.5	0.92	<0.01	8.0±1.9	0.93	<0.01	54± 72	540	<0.01
15% M.S.Th. 85% Chol.	10	4	16.1±4.9	1.36	<0.01	11.2±0.8	1.30	>0.05	246± 69	2460	>0.3
7% M.S.Th. 93% Chol.	0	4	21.3±3.5	1.81	<0.02	10.8±0.5	1.26	<0.02	227± 65	2270	>0.2
Thiouracil-Fed Controls		8	30.0±5.7	2.54		12.9±1.5	1.50		316±112	3160	
Untreated Controls		9	11.8±3.3	1.00	<0.01	8.6±1.1	1.00	<0.01	0.1±0.4	1	<0.01

[1] This is a rough estimation of the dosage. Weight changes in pellets were very small, and numerous factors could markedly influence the final result.

[2] P values are those comparing the group with thiouracil-fed controls.

The daily subcutaneous injection of 8.9 mg. of NaI in rats fed 0.1 thiouracil for 14 days did not reduce the number of intracellular colloid droplets or the cell height of the thyroid (Table 7). Thyroid weight, however, was reduced by about one-third.

7. Are the Adrenal or Its Secretions Responsible for Droplet Formation? Small numbers of colloid droplets were present in 3 of 4 adrenalectomized animals kept at room temperature one day after operation. Adrenalectomy did not prevent an increase in the number of droplets after exposure to 5°C. for 2 hours (Table 6).

Treatment of normal rats kept at room temperature with injections of epinephrine or adrenal cortical extract did not stimulate formation of intracellular colloid droplets (Table 6).

DISCUSSION

Under some conditions, the formation of colloid droplets in the thyroid epithelium of living rats was related to activation by pituitary extract or dependent on the presence of the pituitary gland. Thus, droplet formation in the thyroid of the hypophysectomized rat occurred promptly following the administration of small dosages of pituitary extract. Also, hypophysectomy resulted in the complete disappearance of colloid droplets from the thyroids of animals in whom they had been formed by cold-exposure or thiouracil feeding. Nor did droplets form in the thyroid of hypophysectomized rats after exposure to cold or after thiouracil administration. Further evidence that the formation of droplets in the thyroids of cold-exposed or thiouracil-fed normal rats was mediated through the pituitary is provided by the experiments in which thyroxine, but not iodide, was shown to inhibit this response. This effect of thyroxine was probably due to an inhibition of release of thyrotrophic hormone of the pituitary gland rather than to an interference with the response of the end organ. Thyroxine did not interfere with the response of the hypophysectomized rat to simultaneously administered pituitary extract, but did inhibit the formation of droplets in normal rats exposed to cold or fed thiouracil. This inhibition was apparent as early as two hours after the injection of thyroxine.

The response of the thyroid to cold-exposure was not mediated through the adrenals since it occurred in adrenalectomized rats. Nor was the stimulation of the adrenal cortex by exposure to cold (Long, 1947; Sayers and Sayers, 1947) responsible for the changes in the thyroid. In the dosages used, injections of either adrenal cortical extract or of epinephrine, which is secreted in increased amounts during cold-exposure (Cannon, et al., 1927; Cramer, 1928), did not stimulate droplet formation.

However, droplet formation was not a specific response for thyrotrophic hormones. Droplets formed in the thyroids of hypophysectomized rats following the injection of toxic dosages of typhoid

vaccine or histamine or pilocarpine. Although the reason for it is not clear, this response may be analogous to the spontaneous formation of these structures in vitro in surviving ischemic thyroid tissue (Champy, 1915; Demuth, 1932; Dvoskin, 1947d). Possibly, an ischemia of the thyroid gland in vivo may result following the injection of toxic agents. Or, possibly, these agents may have a chemical irritant effect on thyroid tissue somewhat akin to the effect of mechanical irritation in stimulating droplet formation (Williams, 1944; Dvoskin, 1947d). Whether proteolytic enzymes or inactivated adherent thyrothrophic hormone became activated under these conditions remains undetermined.

The decrease in response to pituitary extract and typhoid vaccine as the interval after hypophysectomy was increased is of interest. Whether this represents a true decrease in sensitivity, or signifies a delay in response to these materials at one hour after injection, with a possible peak response at some later time, is not known. Theoretically, the hypophysectomized rat at 20 days after operation might provide a suitable test object for thyrotrophic hormone, since the non-specific response to typhoid did not occur at this time. However, such an animal responds less to pituitary extract at this time. Also, the counting of droplets is complicated by the paucity of cytoplasm and the plethora of nucleoli, which stain like colloid droplets, in the prominent nuclei of the flattened thyroid epithelium.

The fact that toxic materials may stimulate droplet formation in the thyroid of the hypophysectomized rat one day after operation could explain the disparity of results obtained in attempts to assay the thyrotrophic hormone content of rat as compared with human sera.

Rat sera were never toxic or lethal to the test animal. Even the sera of rats following thyroidectomy were non-toxic. With rat sera, no positive response was ever obtained unless the animal had been thyroidectomized or fed thiouracil. In the presence of an intact thyroid, no positive test was obtained, even after the donor animal had been exposed to cold. Possibly the inactivation of circulating thyrotrophic hormone by the thyroid (Seidlin, 1940; Rawson, Sterne and Aub, 1941, 1942) may explain the absence of detectable amounts of this material in our assay of sera from intact rats. No detectable activity was found in the serum of rats thyroidectomized for short periods. However, thyroidectomy followed by exposure to cold for a short period resulted in the presence in the serum of large amounts of a material stimulating droplet formation. The positive responses secured with serum from normal thiouracil-fed rats is in agreement with the results of Grasso and De Robertis (1946), who used intact rats for assay purposes. Possibly, this is due to the reactivation (Rawson, Albert, McArthur, Merrill, Lennon and Riddell, 1946) or augmentation (Albert, Rawson, Merrill, Lennon and Riddell, 1947)

of thyrotrophic hormone by thiouracil. However, incubation of serum from normal rats exposed to the cold with thiouracil did not result in a positive response in our experiment.

Human sera, on the other hand, were frequently very toxic and lethal to the test animals. Positive responses were also very frequent and were unrelated to the thyroid status of the individual. Among the normal subjects, the positive responses seem related to the toxicity of the sera. Among the other groups, this relationship was less clear-cut. Some of these sera were very toxic, and yet induced but little droplet formation.

Since droplet formation may be induced in hypophysectomized animals with toxic dosages of typhoid vaccine or histamine or pilocarpine or with toxic human sera, though admittedly we may have a pituitary factor present in the case of the sera, one must regard the results of such assays for thyrotrophic hormone using droplet formation with suspicion. This is particularly true when rats with intact pituitary glands are used (Grasso, 1946; Grasso and De Robertis, 1946) since in addition to the possibility of a non-specific response, one has the added possibility of participation of the test aminals' own pituitary gland.

CONCLUSIONS

Under some conditions, the formation of colloid droplets in the thyroid epithelium of living rats was related to activation by thyrotrophic hormone. In hypophysectomized rats, the administration of small dosages of pituitary extract was followed by a prompt appearance of large numbers of droplets. Larger dosages stimulated formation of proportionately larger numbers of droplets. This response was not prevented by the simultaneous administration of thyroxine or iodide. There was a progressive decrease in the number of droplets which formed one hour after the injection of a fixed dosage of pituitary extract as the interval after hypophysectomy was increased. The droplets present in the thyroid epithelium of normal rats exposed to cold or fed thiouracil promptly disappeared following hypophysectomy. Droplet formation was inhibited in normal rats exposed to cold or fed thiouracil by the simultaneous administration of thyroxine, but was not inhibited by administration of iodide. The adrenal or its secretions were not responsible for droplet formation.

Under other conditions, droplet formation was independent of thyrotrophic hormone since droplets appeared in the thyroid epithelium of hypophysectomized rats one day after operation and one hour after the injection of toxic dosages of typhoid vaccine or histamine or pilocarpine. Droplet formation at one hour after typhoid injection was markedly decreased at 8 days after hypophysectomy, and was absent at 20 days after hypophysectomy.

Fresh human sera were often toxic and lethal when injected into hypophysectomized rats. The numerous positive responses which re-

sulted from these injections were unrelated to the thyroid status of the donor.

Fresh rat sera were never toxic or lethal. Positive responses were obtained with sera from thyroidectomized rats or thiouracil-fed rats, and were not obtained under other conditions in the presence of an intact thyroid gland in the donor rat.

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